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Sleep, metabolism, and aging

Thèse de doctorat ès sciences de la vie (PhD)

présentée à la

Faculté de biologie et de médecine
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par

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2. Abstract

Aging is a multidimensional process of physical, psychological, and social changes. Understanding how we sleep and how this dynamic process evolves across life span will help to identify normal developmental aspects of sleep over time and to create strategies to increase awareness of sleep disturbances and their early management. In normal sleepers from HypnoLaus cohort, we evaluated the effects of age and gender on both subjective and objective sleep measurements. Our results indicate that normal aging is not accompanied by sleep complaints, and when they exist suggest the presence of underlying comorbidities. Polysomnographic data revealed that slow wave sleep was more affected with age in men, and age affected differently NREM and REM spectral power densities. Both sleep structure and spectral analysis profiles may constitute standards to delineate pathological changes in sleep, both for aging women and men.

Another important aspect in the management of sleep and its disorders is a detailed characterization of sleep-inducing medications. Gamma-hydroxybutyrate (GHB) is an inhibitory neurotransmitter derivative of GABA, but its mode of action and the range of effects are not well understood. Several properties, as growth hormone stimulation in humans and the development of weight loss in treated patients suggest an unexplored metabolic effect. In different experiments we assessed the effects of acute, short term and chronic GHB administration on central (cerebral cortex) and peripheral (liver) biochemical processes involved in the metabolism of the drug, as well as the effects of the drug on metabolism in C57BL/6J, GABAB knock-out and obese (ob/ob) mice. We showed that GHB treatment affects weight gain in C57BL/6J and GABAB knock-out mice. Metabolomic analysis indicated large central and peripheral metabolic changes induced by GHB with important relevance to its therapeutic use.

3. Résumé

Le vieillissement est un processus multidimensionnel accompagné par de multiples changements dans les domaines physique, psychologique et social. Comprendre comment nous dormons et comment ce processus dynamique évolue sur la durée de vie nous aidera à identifier les aspects normaux du développement du sommeil au fil du temps, et à créer des stratégies pour accroître la connaissance et compréhension des troubles du sommeil et leur prise en charge précoce. Chez les sujets normaux de la cohorte HypnoLaus nous avons évalué les effets de l'âge et du sexe sur les mesures subjectives et objectives du sommeil. Nos résultats indiquent que le vieillissement normal ne s'accompagne pas de troubles du sommeil, et quand ils existent ceux-ci suggèrent la présence de comorbidités sous-jacentes. Les données polysomnographiques ont révélé que le sommeil profond était plus affecté avec l'âge chez les hommes. De plus, nous avons montré comment l'âge modifie la composition spectrale du sommeil lent et paradoxal. La structure du sommeil et les profils d'analyse spectrale peuvent donc constituer des standards permettant de définir les changements pathologiques du sommeil chez les personnes âgées.

Parmi les aspects importants de la gestion du sommeil et de ses troubles, la caractérisation détaillée des médicaments hypnotiques utilisés est essentielle. L'acide gamma-hydroxybutyrique (GHB) est un acide gras à courte chaîne dérivé du GABA, principal neurotransmetteur inhibiteur du cerveau, mais son mode d'action et tous ses effets sont toujours largement méconnus. Plusieurs propriétés, comme la stimulation de la sécrétion de l'hormone de croissance chez l'homme et le développement d'une perte de poids chez les patients traités suggèrent un effet métabolique inexploré. Dans différentes expériences, nous avons évalué les effets d'une exposition aiguë, à court terme et chronique de GHB sur les processus biochimiques centraux (cortex cérébral) et périphériques (foie) impliqués dans le métabolisme du médicament. Nous

avons aussi évalué les effets du médicament sur le métabolisme des souris C57BL/6J, GABAB KO et obèses (ob/ob). Nos résultats ont montré que le GHB diminue le gain de poids chez les souris C57BL/6J et GABAB KO. L'analyse métabolomique a indiqué des changements importants induits par GHB au niveau central et périphérique, et ces effets sont importants pour son utilisation thérapeutique.

4. Résumé pour un large public

Notre sommeil change continuellement, depuis la naissance jusqu'aux âges avancés. Les personnes âgées se plaignent souvent de la qualité ou de la quantité de leur sommeil mais les causes restent inconnues. Les maladies du sommeil augmentent aussi chez les personnes âgées ; cependant nous ne savons pas si ce phénomène est dû à une dégradation normale du sommeil ou à d'autres facteurs. Un autre aspect important est l'utilisation des hypnotiques, très fréquente chez les seniors avec des effets secondaires plus ou moins importants.

Nous avons voulu savoir si le sommeil physiologique change en fonction de l'âge, en dehors de toute pathologie connue du sommeil. Nous avons aussi voulu savoir si ces changements sont différents en fonction du sexe des individus. Pour ce travail, nous avons analysé des questionnaires relatifs au sommeil de 2211 individus et les enregistrements du sommeil de 890 d'entre eux. Cette population appelée HynoLaus est représentative de la population générale lausannoise. Nous avons aussi étudié les conséquences métaboliques de Gamma-hydroxybutyrate (GHB), une substance hypnogène utilisée dans le traitement de plusieurs maladies. Cette étude a été menée chez les souris.

Nos travaux indiquent que la durée du sommeil ne change pas avec l'âge. Contre toute attente, nous avons aussi trouvé que les individus les plus âgés se plaignaient moins de la qualité de leur sommeil et se sentaient moins somnolents dans la journée. Les enregistrements de sommeil indiquent des changements significatifs dans la structure du sommeil avec un effet plus marqué chez les hommes. Ces résultats indiquent que les plaintes de sommeil chez les personnes âgées ne sont pas dues aux changements physiologiques du sommeil et doivent être investiguées systématiquement pour en trouver les causes.

Nos études pharmacologiques chez la souris indiquent que le GHB induit des changements majeurs dans le métabolisme, aussi bien cérébraux que périphériques. Ces changements sont responsables d'une diminution de poids et d'une meilleure répartition des sources d'énergie. Nous avons aussi démontré que le GHB avait des effets antioxydants et favorisait la disponibilité des acides gras oméga-3. Nos résultats suggèrent que ces effets métaboliques ne seraient pas d'origine cérébrale mais périphérique.

Les résultats obtenus pendant ce travail de doctorat constituent une base normative pour le sommeil en fonction de l'âge et du sexe. Nos travaux ont aussi permis de comprendre comment un hypnotique peut, en dehors de ses actions centrales, modifier le métabolisme général.

5. Introduction

« Chercher n'est pas une chose et trouver une autre, mais le gain de la recherche c'est la recherche même ».
Saint Grégoire de Nysse

Complexity of sleep and its interconnection with all other biological functions partly explains why, after almost one century of sleep research, basic questions are still unanswered. Fascinating and intriguing, this state of apparent unresponsiveness to external stimuli and reduced information processing is still waiting for scientist to discover "why" starting from "how". The detailed steps and mechanisms by which different neurotransmitters (acetylcholine, histamine, noradrenaline, dopamine, serotonin, orexin, gamma-aminobutyric acid, adenosine, etc) are controlling and regulating sleep are yet to be discovered. Nevertheless, it is known that the alterations in sleep duration or structure, or the acute or chronic lack of sleep have important impacts on most other biological functions in terms of metabolism [1-9], brain energy[10-15], cardiovascular response[16-30], immune function[31-47], performance[48-59] and many other functions[17,60-70].

Like all major governing processes within our body, sleep is evolving with age, affecting its three major characteristics: timing, duration and quality[71]. While normal structure of sleep in children and during adulthood is well established and standards are available, normative data for normal sleep in aging are missing. Studying sleep in older subjects is challenging because of important overlapping of comorbidities, which can impact sleep. Since deviations from normal patterns are often subject to interventions, characterization of normal sleep in aging population becomes of increasing importance. Moreover, description of modifications in sleep structure in different age groups represents the first step in finding the substrates involved. Published studies

indicate changes in sleep architecture[72-97] such as increase in wake after sleep onset and stage 1 NREM sleep on the expense of slow wave sleep (SWS, Stage 3) and REM sleep, but these changes *per se* are not always associated with subjective changes in sleep quality[98,99] or daytime sleepiness measured by Epworth Sleepiness Scale. Attempts made to discover genes related with sleep duration[71] showed, for example, that an intronic variant in *ABCC9* gene explains about 5% of sleep duration in unrelated subjects. Twin studies showed significant heritability estimates for sleep length, quality and variations in sleep patterns[100]. Pharmacogenetic studies identified genetic variations involved in different responses to wake-promoting medications.[101] Inbred mice studies showed that genetic contribution is stronger for the EEG spectral composition than for sleep duration and structure.[100] Given the complexity of sleep and wakefulness, it is likely that both intrinsic and extrinsic factors participate in the modulation of sleep architecture with aging.

Moreover, aging is associated with increase in the prevalence of sleep disorders, especially insomnia[102], obstructive sleep apnea[103] and restless leg syndrome.[104] All sleep-related disorders alter significantly sleep structure, but their higher prevalence in elderly might suggest that age-related changes in sleep structure facilitate the development of these disorders.

An important factor affecting the modulation of sleep with aging is gender. Previous studies reported poorer subjective sleep quality in women[105], but polysomnographic data showed that they have a better sleep structure[81] in terms of SWS amount and wakefulness after sleep onset[106]. It was proposed that different psychosocial factors in men and women may contribute to dissimilarities in the perception and evaluation of symptoms[106] and this may contribute to the differences observed in self-rating sleep quality. Women usually complain about difficulties in falling asleep[107] and this is consistent with increased prevalence of insomnia with aging in women[108], while men have more problems in sleep maintenance, lighter sleep and more frequent sleep related breathing disorders[109]. Gender differences in sleep were mostly attributed to

hormones, but also social and cultural factors might participate[110]. Other studies offer contradictory gender differences, with men complaining more about their sleep[111]. Even if there is an important amount of published work on the evolution of sleep with age, the large variability in methodology, sample size, or design are just a few reasons why for the same hypothesis the results are discordant.

More recent results pointed to gender differences in the sleep EEG. If some studies concluded that ageing in men and women are similar[112], other showed that disentangling the effect of gender on EEG spectral composition might be confounded by methodological issues[113]. It was proposed that men have lower spectral power in delta-theta frequency bands[114], but different analysis showed the opposite effect of gender especially depending on the environment, which may explain the fragility of women's sleep in disturbed environments[115].

Increase in demand for sleep-inducing medication, especially in older populations, to overcome longer sleep latency or altered sleep maintenance raises the need to discover pharmacologic tools which can mimic physiological sleep. Different neurotransmitters are involved in the induction and generation of sleep. Gamma-hydroxybutyrate, a gamma-aminobutyric acid agonist, has proven hypnotic properties. Of special interest is to investigate central effects of this drug, aiming to identify its mode of action. Additionally, very few data are available on its peripheral effects or consequences on metabolism.

The first aim of my PhD project was to characterize sleep patterns and main EEG features, as well as the subjective impact of changes in sleep dynamic on the daytime functioning in relationship with age and gender in a cohort of normal sleepers representative of the general population of Lausanne. Investigating the main differences in sleep in subjects without sleep complaints will contribute to the understanding of the substrates responsible and of their functional significance and establish the normative data on sleep in aging populations.

The second part of my project was designed to investigate the metabolic effects of sodium oxybate (gamma hydroxybutyrate, GHB), a drug used in sleep medicine to treat narcolepsy with cataplexy. If the efficacy of this drug in treating narcolepsy patients is established[116 117] and the impact of the drug on sleep in healthy subjects is described[118], the metabolic changes induced in narcolepsy patients gained attention only recently[119-122]. Furthermore, the distribution of endogenous GHB is not limited to the nervous system. It is normally present in other tissues such as the kidney, heart and skeletal muscle, with markedly greater concentrations than in the brain[123] and might have several actions. The goal of this part was to identify the metabolic pathways affected by acute and chronic administrations of the drug in mice. Since narcolepsy patients are chronically treated with GHB, we investigated also how long-term administration of the drug affects body weight and body composition in different mouse strains.

Before the detailed presentation of these two projects, the sleep structure, neurobiology and regulation, the relationship between sleep and aging, and sleep pharmacology with special emphasis on GHB will be summarized.

5.1 Sleep architecture

The gold-standard tool for recording and analyzing sleep in humans and other mammals is polysomnography (PSG). This method allows collecting physiologic information on cerebral cortex activity, muscle tone, eyes movements, respiration, and cardiovascular activity during sleep. Based on electroencephalographic signals altogether with eye movement and muscle tone, sleep stages are scored according to criteria published almost 50 years ago by Rechtschaffen and Kales and revised in 2007 by AASM.[124]

Sleep is divided into two major states: non-rapid eye movement (NREM) sleep and rapid eye movement (REM) sleep which alternate cyclically. NREM sleep has three distinct stages: stage 1, stage 2 and stage 3, each stage having distinct electroencephalographic (EEG) features. Stage 1 NREM sleep has a transitional role, is shallow and occupies about 5% of the total sleep amount. This stage is characterized by low EEG amplitude, and the dominating frequencies are theta (4.75-8 Hz) and alpha (8.25-11 Hz); vertex sharp waves and slow eye oscillations may be present during this stage. Stage 2 NREM sleep is defined by the presence of special EEG features, such as K-complexes and spindles and represent 45-50% of total sleep time. 25% of NREM sleep is represented by the deepest stage of NREM sleep, slow wave sleep (SWS, stage 3). The dominant frequency in SWS is delta (0.75-4.5 Hz), with high amplitude and slow frequency waves. REM sleep is defined by wake-like and "activated" (high-frequency, low-amplitude) EEG[125], muscle atonia and rapid eye movements. The main EEG pattern of this sleep stage is represented by "saw tooth" waves.

The evolution of sleep stages across the night is not random. During the night there are four to five NREM-REM sleep cycles (80-100 minutes each), with the two states alternating. At the beginning of the night, NREM is deeper and longer, while REM sleep is shorter or even

absent. As the night progresses, REM sleep duration increases and NREM sleep becomes lighter and shorter (Figure 1).

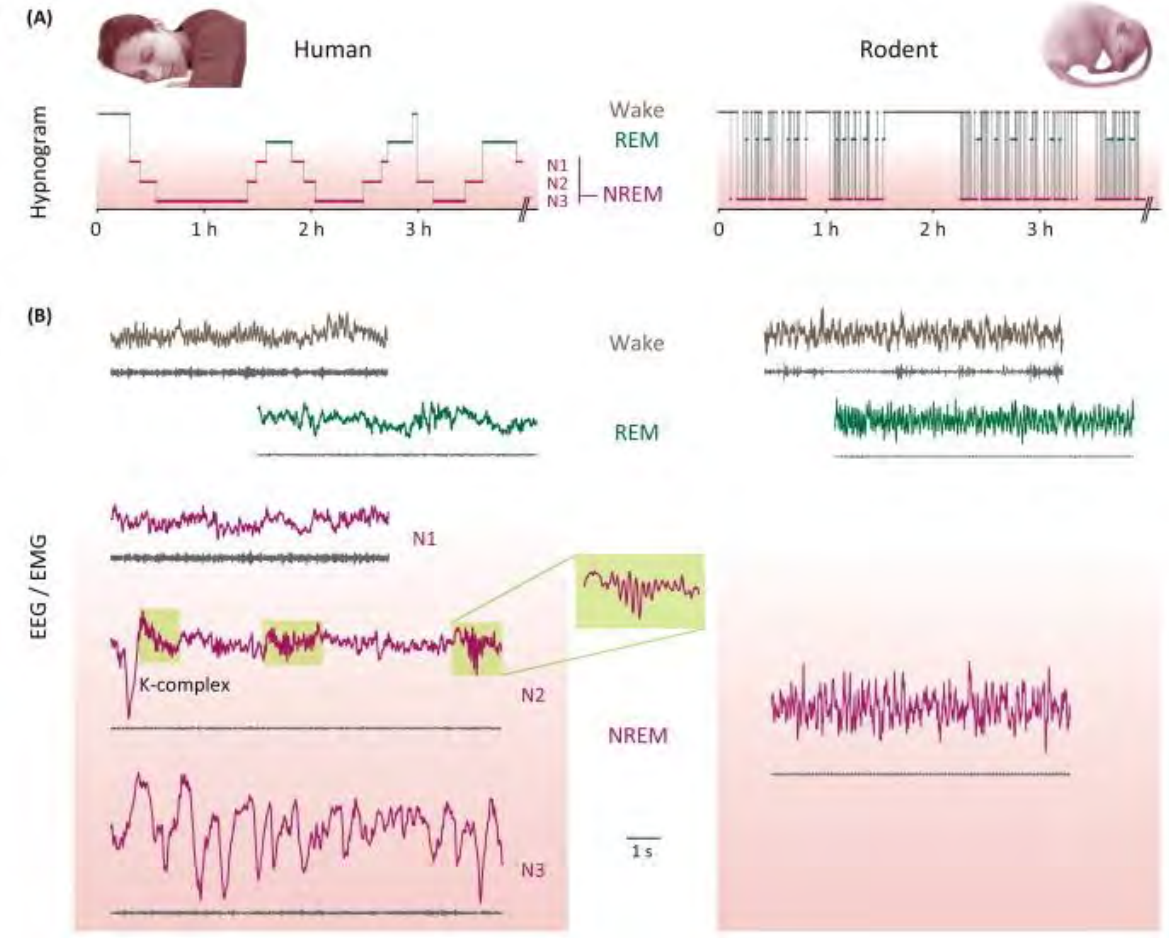

Figure 1. *Hypnogram (A) and polysomnographic characteristics for each sleep stage (B). NREM sleep is divided in three different stages with stage 3 (SWS or N3) being the deepest. EEG and electromyogram (EMG) for each state illustrate the main features of each sleep state. Specific EEG patterns for stage 2 (K-complex and spindles) are outlined in green (modified from reference [126]).*

5.2 Neurobiology of sleep and wakefulness and sleep regulation

The development of the two-process model of sleep regulation[127-129] was one of the most important advances in understanding the regulation of sleep-wake system. As a result of interactions between a circadian (sleep independent, dependent on circadian oscillator) and a homeostatic (sleep and waking dependent), two distinct but complementary processes, sleep and

wakefulness alternate in a specific manner. Sleep propensity is assumed to be the result of the combined action of these two processes[128].

The timing and the organization of sleep architecture are strongly influenced by the circadian system (process C), while sleep need and intensity are homeostatically regulated. The circadian and homeostatic processes contribute equally to the sleep consolidation and wake performance[130]. The circadian pacemaker, with intrinsic oscillatory properties, is situated in the suprachiasmatic nucleus which, under normal circumstances, is reset on a daily basis by external time cues, the most important being light inputs from the retina (melanopsin photosensitive ganglion cells, via retinohypothalamic tract) during the day, and by melatonin secretion from the pineal gland during the dark period[131]. The circadian system mediates rhythmic outputs to sleep-wake activity and to all functions of the body and is connected with neurons involved in the maintenance of wakefulness and in the generation of NREM and REM sleep[131]. Recent animal studies showed that the circadian system influences the amount of vigilance states and also the intensity of NREM sleep, actively promoting wake or sleep at opposite phases of its cycle[132,133]. The signals sent by the circadian clock to maintain wakefulness attenuate after melatonin secretion. During wakefulness, sleep pressure (or sleep need) progressively builds up. Sleep homeostasis (process S) regulates the amount and intensity of sleep as a function of prior wakefulness. The best EEG indicator of sleep intensity is delta power (0.75-4.50 Hz) while theta-alpha power (6.25-9.00Hz) increases as waking accumulates and is associated with the homeostatic build-up of sleepiness[134,135].

The discovery of main anatomical structures and neurotransmitters involved in sleep or wake generation and maintenance dates back to the beginning of the 20th century, when von Economo reported a different type of encephalitis associated with very long sleep duration. In these patients, von Economo identified lesions at the junction of midbrain and diencephalon. He

proposed that these structures are part of an arousal system that keeps the brain awake. Decades after, Moruzzi and Magoun[136] described the ascending arousal pathway which they named ascending reticular activating system (ARAS). ARAS is originating in the brainstem and send projections through reticular formation of the midbrain. Further studies identified two arousal pathways (a dorsal and a ventral) contributing to the wake-sleep alternation. The ventral arousal pathway starts from upper brainstem and includes projections from reticular formation (RF) to the basal forebrain (BF), posterior hypothalamus, and cortex. These projections originating from the locus coeruleus (LC - noradrenergic), dorsal and median raphe (serotoninergic), brainstem cholinergic nuclei, and mesencephalic dopaminergic nuclei, arouse the cortex both directly and through the thalamus. The ventral arousal pathway receives inputs from basal forebrain cholinergic, tuberomammillary histaminergic, and lateral hypothalamus (melanin concentrating hormone neurons and orexin neurons) and project diffusely to the cortex.

Wakefulness – cortical activation

Cholinergic neurons located in RF, pedunculo-pontine and laterodorsal tegmental nuclei project to the BF and thalamus, and those located in the BF project to all cortical areas. Noradrenergic neurons located in the LC (the most prominent noradrenergic nucleus) and serotonergic raphe neurons send projections to the thalamus and the cortex. Dopamine containing neurons from the ventral tegmental area and substantia nigra project to BF and cortex, and histamine producing neurons from tuberomammillary nuclei (located in the posterior hypothalamus) project diffusely to the cortex. The excitatory neuropeptides orexin A and B are produced by the neurons in the lateral and posterior hypothalamus and are involved in sustaining wakefulness. They project to the cortex, BF and brainstem, and their excitatory properties are exerted on acetylcholine, histamine containing hypothalamic neurons, noradrenergic, and

serotonergic neurons. Glutamate and aspartate, two excitatory aminoacids, are contained in many neurons located in subcortical regions and cortical pyramidal cells, which project widely to the cortex, forebrain and brainstem. Wakefulness and cortical activation can be maintained by other neuromodulatory substances such as: vasoactive intestinal peptide, corticotropin-releasing factor, thyreotropin-releasing factor, thyreotropin-stimulating hormone, adrenocorticotropic hormone and adrenaline. Arousal systems, apparently redundant, are independently able to promote wakefulness, but they interact in order to generate behavioral arousal and all excite thalamic and cortical neurons[137]. The EEG markers of arousal are high theta and gamma oscillations. Descending projections of neurons from arousal systems impact the sensorial and motor responsiveness, muscle tone and other physiological functions[138].

NREM sleep

von Economo also observed that encephalitis patients with lesions in preoptic area and BF presented with insomnia, suggesting these neurons are involved in sleep generation. Two regions situated in preoptic area (ventrolateral preoptic or VLPO and median preoptic area or MNPO) contain GABA and galanin releasing neurons and start firing during NREM sleep (VLPO) or during both NREM and REM sleep (MNPO) and inhibit many arousal nuclei: LDT/PPT, LC, DR, TMN, and orexin neurons (Figure 2). MNPO neurons are involved in the initiation of NREM sleep, while the main role of VLPO neurons is to maintain NREM sleep. GABA containing neurons were identified also in the thalamus, where they inhibit thalamocortical relay neurons. Besides GABA, an important role in initiation of NREM sleep is played by adenosine, which inhibits wake active neurons from BF through A1 receptors, contributing to the initiation of sleep[139]. Two main EEG features characterize NREM sleep: spindles and delta waves. Synchronization of cortical activity is facilitated by projections from thalamus. The feedback

loop between two circuits – thalamocortical (excitatory aminoacids) and reticulothalamic (GABAergic) – results in spindles production. When thalamocortical neurons reach a certain level of inhibition they change to an intrinsic firing mode, leading to the generation of SWS.

REM sleep

Within a sleep cycle, a NREM sleep episode is followed by a REM sleep episode. This complex sleep state is generated in the brainstem, is actively promoted by cholinergic neurons and suppressed by serotoninergic or noradrenergic neurons. Besides EEG specific features, rapid eye movements and muscle atonia differentiate REM sleep from NREM sleep or wakefulness. Two types of neurons were described: REM-on and REM-off neurons, based on their firing during this state. An important site for the generation of REM sleep is perilocus coeruleus, with GABA and glutamate containing neurons, but recent observations suggest an important role of GABA, glutamate, glycine and neurons producing melanin concentrating hormone (MCH) in the regulation and maintenance of REM sleep.

Even if numerous neuronal structures are involved in the precise alternation between sleep and wakefulness, none is sufficient or necessary. Pathological alteration, genetic manipulations, or pharmacological interventions in each of these pathways affects the initiation, duration, and the quality of the vigilance states, with significant impact on the quality of life, but none can completely abolish any vigilance state. [137]

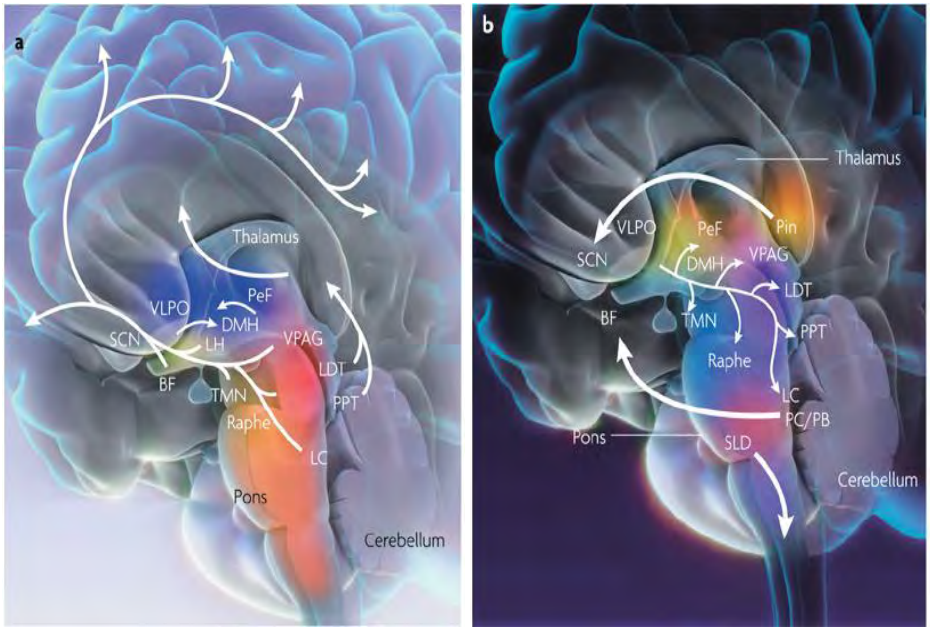

Figure 2. *Main pathways involved in arousal (a) and sleep (b). An important contribution to wakefulness is represented by cholinergic inputs from the brainstem projecting to the thalamus which then activates the cortex (laterodorsal tegmental [LDT]; pedunculopontine nuclei [PPT]). Moreover, thalamocortical pathways participate to wakefulness maintenance through noradrenergic (locus coeruleus [LC]), serotoninergic (dorsal raphe [Raphe]), histaminergic (tuberomammilary nucleus [TMN]), dopaminergic (ventral periaqueductal grey matter [VPAG]) and orexinergic (from perifornical neurons [PeF]) inputs.NREM is initiated by GABAergic and galanin inhibitory signals (from the ventrolateral preoptic nucleus [VLPO) to arousal centers. REM sleep is controlled by cholinergic and non-cholinergic projections from the brainstem, and REM sleep atonia is mediated by glutamatergic (from sublaterodorsal nucleus [SLD]) and glycinergic projections to the spinal cord (figure from [140]).*

5.3 Main EEG sleep characteristics

The analysis of sleep stage duration and distribution is rather limited nowadays, therefore transformation of EEG signal by fast Fourier transform is used to assess the contribution of EEG frequency composition and density of different frequency bins. Main EEG rhythms used to characterize sleep are delta, theta, spindles and beta waves. Delta rhythm or delta power (0.5-4.5Hz) is used as a marker of sleep need and intensity, since the power in this frequency range increases after sleep deprivation and decreases exponentially across the night. Delta waves have both cortical and thalamic origins, and the highly synchronized activity is the result of cortico-cortical and thalamocortical interplay. Also, slow oscillations (<1Hz) have entirely cortical origins[141]. Studies in animal models and in humans showed that slow oscillations have an anterior

frontal origin and after sleep deprivation the increase in delta power is larger in this area. These oscillations propagate fast to the posterior parts of the brain[141]. If delta activity is the marker of sleep propensity, EEG theta oscillations (4-8Hz) characterize wakefulness quality, being a good indicator of sleep pressure during extended wakefulness. Spindles are waxing-and-waning oscillations, lasting 0.5–2s, a hallmark of non-NREM sleep[142]. Based on EEG frequency, region and origin, two different types of spindles were described: slow spindles (10-12Hz), mostly present in the frontal cortical areas, and fast spindles (12-15Hz), which predominate in the central and parietal regions. [143] Alpha activity (8-10Hz) was proposed as a marker of REM sleep homeostasis, due to its attenuation in REM sleep after both total or selective REM sleep deprivation[144]. Also, across and within REM sleep episodes, alpha activity decreases. During NREM sleep, alpha activity is also decreased[145]. It was suggested that fast rhythms (beta and gamma) are generated by the cortical neurons projecting to thalamus[141]. During wakefulness they were correlated with cognitive processes while during REM sleep they were associated with dreaming, although they are also present in NREM sleep. The main model explaining the difficulties in initiating and maintaining sleep is based on the cortical hyperarousal model[146], and beta frequency activity was found increased during both NREM and REM sleep in insomnia patients.[147]

5.4 Sleep and aging

Age-induced changes in sleep affect its duration, timing, and quality. Aging affects not only the duration and the architecture of sleep, but also the EEG features of sleep.[148] Important gender differences accompany most of these modifications across the life span, but the exact relationship with gender is largely unknown[112]. Not all studies support the decrease in sleep duration with aging, but they agree on most architectural changes with proportional increase in

lighter stages of sleep, more stage shifts, and less deep sleep[149]. The decrease in sleep depth, intensity, and continuity is also accompanied by a reduced amplitude of circadian rhythm output signals[150]. Reduced sleep efficiency and state instability are thought to be determined by increased brain arousal during sleep and decreased ability to maintain NREM sleep[151,152]. Also, during NREM sleep, the arousal threshold is diminished in elderly, especially in women[115]. This decrement in NREM sleep stability might be correlated with decreased spindle acitivity[153]. Note that although older subjects have lower absolute SWS levels, the homeostatic response to increasing sleep need is basically operational[150].

Circadian system is also affected in aging with a circadian phase advance, attenuated amplitude of circadian markers such as melatonin, core body temperature[154] and cortisol[155], all of which can be linked to changes in sleep structure. Nevertheless, age-related changes in the circadian physiology are substantially different between individuals based on their genetic background, as for instance a polymorphism in *PER3* gene, is associated with different responses to sleep deprivation and performance[156].

Sleep alteration observed in humans could be seen also in old mice and rats. [157,158]. Deterioration of the orexin system [158,159], ageing-related decline in adenosine A1 signaling [160], and cholinergic hypofunction in the basal forebrain[161] may play a role in sleep changes associated with aging. Nevertheless, age-related alterations might not be the result of loss of critical neurons, but to more subtle changes in cell-cell interactions in brain regions involved in sleep-wake control[162]. Moreover, recent mouse studies showed that age-related changes in sleep architecture and EEG activity are strongly under a genetic control.[163]

The first subjective outcome of inadequate or insufficient sleep is sleepiness, which can be measured by using questionnaires, and objectively, by multiple sleep latency test (which measures the time needed to fall asleep) or maintenance of wakefulness (which measures the

ability to stay awake). Published studies are discordant concerning sleepiness in aging subjects, with some reporting increased[164] and others reduced daytime sleepiness[92]. Even if sleep disorders, an important cause of sleepiness, may not be part of the normal healthy aging, their presence should be identified and managed accordingly.

Subjective evaluation of sleep and sleep disorders

In clinical practice, sleep-related questionnaires are commonly used to evaluate the effects of disturbed nocturnal sleep on daytime activity and quality of life, and to screen for the presence of sleep disorders. The most widely used questionnaire to evaluate the presence of excessive daytime sleepiness is Epworth Sleepiness Scale (ESS), which evaluates the chance of falling asleep in 8 different situations. A final score higher than 10 is suggestive for pathological daytime sleepiness (score range: 0-24)[165]. Karolinska sleepiness scale (KSS) is a single item scale used to measure subjective sleepiness at a specific time point. Possible scores range from 1 to 9. Higher scores represent higher subjective sleepiness. The KSS scores were highly correlated with polysomnographic measures of sleepiness[166]. In the general population, aging was found associated with less daytime sleepiness[167,168], and it was suggested that subjective sleepiness has a significant heritable component.[169] Pittsburgh Sleep Quality Index (PSQI) evaluates sleep quality on seven domains[170]: subjective sleep quality, sleep latency, sleep duration, sleep efficiency, sleep disturbances, use of sleep medication, and daytime dysfunctioning over the last month. For each domain the score ranges between 0 and 3, where 3 is the negative extreme. A total score higher than 5 (out of maximum 21) is associated with poor sleep quality. Longitudinal studies using PSQI showed its ability to predict the development of sleep disturbances or sleep complaints with aging. [171]

One of the first questionnaires used to assess chronotype was Horne and Ostberg Morningness-Eveningness questionnaire (MEQ), which asks for time preference for different activities, and sleep preferred schedules. Later, Munich Chronotype was developed, which differentiate sleep timing during working days and week-ends.[172] Age is the major factor affecting MEQ scores, with morningness increasing with aging. Also, the association between *PER3* polymorphism and diurnal preference is strongest in young subjects.[173] Restless leg syndrome rating scale was elaborated by International RLS Study Group, and in ten questions evaluates the severity of the disorder altogether with the impact on subjects daily life and mood.[174] The final score allows scaling of disease severity from mild to very severe. Berlin questionnaire is designed to evaluate the risk of obstructive sleep apnea syndrome, addressing the presence and frequency of snoring, witnessed apneas, daytime sleepiness, obesity or hypertension.[175] Restless leg syndrome and obstructive sleep apnea syndrome have higher prevalence with aging, both with important consequences on sleep quality and health outcomes. [176] The 11 items instrument, Ullanlina narcolepsy scale is used to measure the symptoms of narcolepsy.[177] With a cut-off of 14, this scale is very well discriminating narcolepsy patients from obstructive sleep apnea, multiple sclerosis and epilepsy. Munich Parasomnia Screening is a self-rating instrument based on 21 items, which asses the presence at any time during life and the frequency of parasomnias in adults.[178] It has high specificity and sensitivity for both psychiatric patients and healthy participants. Almost all questionnaires described above were completed by the HypnoLaus participants, but only outcomes from ESS, MEQ and PSQI will be discussed in section 7.

5.5 Sleep pharmacology

"Coming back to where you started is not the same as never leaving."

Terry Pratchett

Up to now, different drug classes, which act on major neurotransmitters involved in sleep, are used to treat sleep disorders. Benzodiazepines which act by enhancing the γ-aminobutyric acid A ($GABA_A$) receptor function in the central nervous system increase sleep duration and decrease stage 1, REM sleep duration, and latency[179,180]. Z-drugs were shown to have fewer side effects than benzodiazepines, decrease sleep onset latency and REM sleep[181]. Antihistaminics are often used as self-administrated sleep promoting medication, but due to their concurrent anticholinergic properties, side effects are common[182]. Another pathway contributing to wakefulness, serotonergic pathway, was explored, and it was shown that nonselective serotonin 5-HT(2A/2C) receptor antagonists, selective 5-HT(2A) receptor antagonist and the 5-HT(2A) receptor inverse agonists increase SWS in both normal sleepers and in patients with chronic insomnia[183]. EVT 201, a partial $GABA_A$ receptor agonist, was shown to decrease sleep onset latency and wake after sleep onset (WASO) in elderly primary insomnia patients[184]. Pregabalin, an antagonist of neuronal calcium ion channels, currently approved for epilepsy, postherpeutic neuralgia [185], neuropathic pain[186], fibromyalgia[187] and generalized anxiety disorders[188] was shown recently to improve insomnia symptoms in fibromyalgia patients[189]. More recently, dual hypocretin antagonsits are emerging as new hypnotics [190].

Symptomatic treatment is applied also in sleep disorders such as idiopathic hypersomnia and narcolepsy, characterized by excessive daytime sleepiness and irresistible sleep episodes. To promote wakefulness in patients with central hypersomnia, standard recommended treatment is Modafinil (or its R-enantiomer with a longer half-life), a non-amphetamine stimulant which binds to dopamine and noradrenaline transporter, inhibiting their action and increasing the extracellular

level of these catecholamines.[191] Amphetamine, methamphetamine, dextroamphetamine, and methylphenidate modulate the level of dopamine and, to a lesser extent, of noradrenaline and their efficacy in treating daytime sleepiness was proven. Manipulation of other neurotransmitters allows re-establishing, on a daily basis, of the balance between sleep and wakefulness. For example, 5-HT(2C) receptor antagonist, agomelatine, is expected to increase arousal[192].

5.6 Gamma-hydroxybutyrate (GHB)

GHB is a unique $GABA_B$ receptor agonist, with a variety of dose-dependent behavioral effects, ranging from euphoria and anxiolysis to deep sleep. The increase in SWS observed after GHB administration in animals and humans attracted attention on this specific molecule, because the positive modulation of SWS is not observed with other sleep-inducing drugs.

Sodium oxybate (Xyrem®, sodium salt of gamma-hydroxybutyrate) is, as from 2007, standard recommendation for the treatment of cataplexy and excessive daytime sleepiness in narcolepsy with cataplexy patients[193]. The efficacy of GHB on main features of narcolepsy – cataplexy and excessive daytime sleepiness – is well established[194]. The greatest reduction in cataplexy frequency was obtained after two weeks of treatment, and was correlated with the dose. [195]. Also, daytime unintended sleep duration decreased, and night sleep architecture was significantly improved, with less stage 1 NREM sleep, longer SWS and less fragmentation. [196,197]. Data on the effects of GHB treatment on hypnagogic hallucinations and sleep paralysis are inconsistent[195]. Nevertheless, GHB treatment has some well-known side-effects: nausea, vomiting, dizziness, or transient events as enuresis, and groaning. *De novo* central apnea[198] and deterioration of sleep-related breathing disorders in obstructive sleep apnea patients[199] were also reported.

GHB is not only used for the treatment of narcolepsy with cataplexy. Alcohol withdrawal syndrome (AWS), mediated by reduced GABAergic activity, may be treated with GHB. The mechanism by which GHB reduces AWS symptoms is believed to be the results of indirect activation of GABAA receptors by GHB. Recent studies demonstrated that GHB was more effective than diazepam in treating AWS[200], improved the abstinence rate in reducing craving for alcohol[201] and maintenance of long term abstinence from alcohol[202].

In recent years GHB was investigated in placebo-controlled clinical studies for the treatment of fibromyalgia. One year of GHB treatment was associated with clinically relevant improvements in pain and daytime functioning, which were maintained during 52 weeks of treatment[203]. GHB improved nocturnal sleep and reduced the intensity and frequency of headaches in four patients with chronic cluster headache, with long-standing effects, but the mechanism is unknown[204]. Due to the small sample size, double-blind randomized controlled studies are needed for confirming the efficacy of GHB in cluster headache treatment. Interestingly, altered human mitochondrial metabolism may affect GHB treatment tolerability[205].

History

GHB was synthetized for the first time by H. Laborit in 1960, who was trying to produce a GABA-derivative able to penetrate blood-brain barrier.[206] Soon after the development of GHB, some of its properties, as sedation and relaxation, were discovered and GHB began to be used as an anesthetic. Intravenous (IV) administration of GHB (60-70 mg/kg) in more than 5000 patients allowed Laborit to detail benefic effects of this atypical anesthetic, like muscle relaxant, absence of respiratory depression, facilitation of induction and maintenance of hypothermia, but also decreased serum potassium level, deficient control of pain and difficulties in predicting the duration of drug induced state[207].

In 1964, Laborit showed that GHB was beneficial in obstetrics due to the absence of respiratory depression in newborn and to the important dilatation of the cervix[206]. Its effects on normal labor were already noted, but in 1968 Tunstall recommend it also for caesarean section, due to the lack of negative effects on the fetus.[208] Just before its release in Great Britain, available data showed that GHB acts as a basal hypnotic, acting primarily on the cerebral cortex, with a peak detected in plasma at 40 min and 140 min in CSF after IV injection of 70 mg/kg[207]. In spite of the lack of analgesic effects, the differences in EEG pattern compared with barbiturates, the increase in amplitude and frequency of uterine contractions, altogether with anti-arrhythmic and anti-anoxic effects, indicated GHB as safe and less toxic alternative to cocktails used in anesthesia, even for children.[207] Potential metabolic effects of GHB, as modification of post-operative catabolism, hypokalemia and hypoglycemia, were also reported.[209] Because of weak analgesic effects and dose-dependent development of seizures in some cases, the drug was precluded from use as an anesthetic agent in most countries. At the beginning of 1980s body-builders started to self-administrate GHB to increase muscle mass[210] and there are also some reports of GHB use as weight reducer,[211]. GHB is used as recreational drug, and at doses of 20-30 mg/kg induces euphoria and amnesia[212]. Doses higher than 50-60 mg/kg may result in coma, which persists for short time and is followed by uneventful recovery[212]. Intoxications due to GHB overdose (or simultaneous alcohol and GHB) present with aggressive behavior, amnesia, vomiting, bradycardia, respiratory depression, coma and even death[213].

Metabolism of GHB

GHB exists naturally in very small concentration in mammalian brain, derived from conversion of GABA, and also in other tissues like heart, kidney and muscle. Metabolism of GHB is described in Figure 3. Main pathway of GHB degradation is through GHB dehydrogenase, which has as a final breakdown product succinic semialdehyde. Succinic

semialdehyde is either converted to succinate, which is further degraded into Krebs cycle, or is transformed into GABA, by GABA transaminase, although results regarding increase in GABA after GHB administration are contradictory[214]. Two other degradation pathways were also proposed: beta-oxidation with glycolate as the final breakdown product, and dehydrogenation by 2-hydroxyglutarate transhydrogenase. The latest enzyme has a poorly understood general function but is active in the nervous system and peripheral tissues, and its final product is 2-hydroxyglutarate.[212,215]

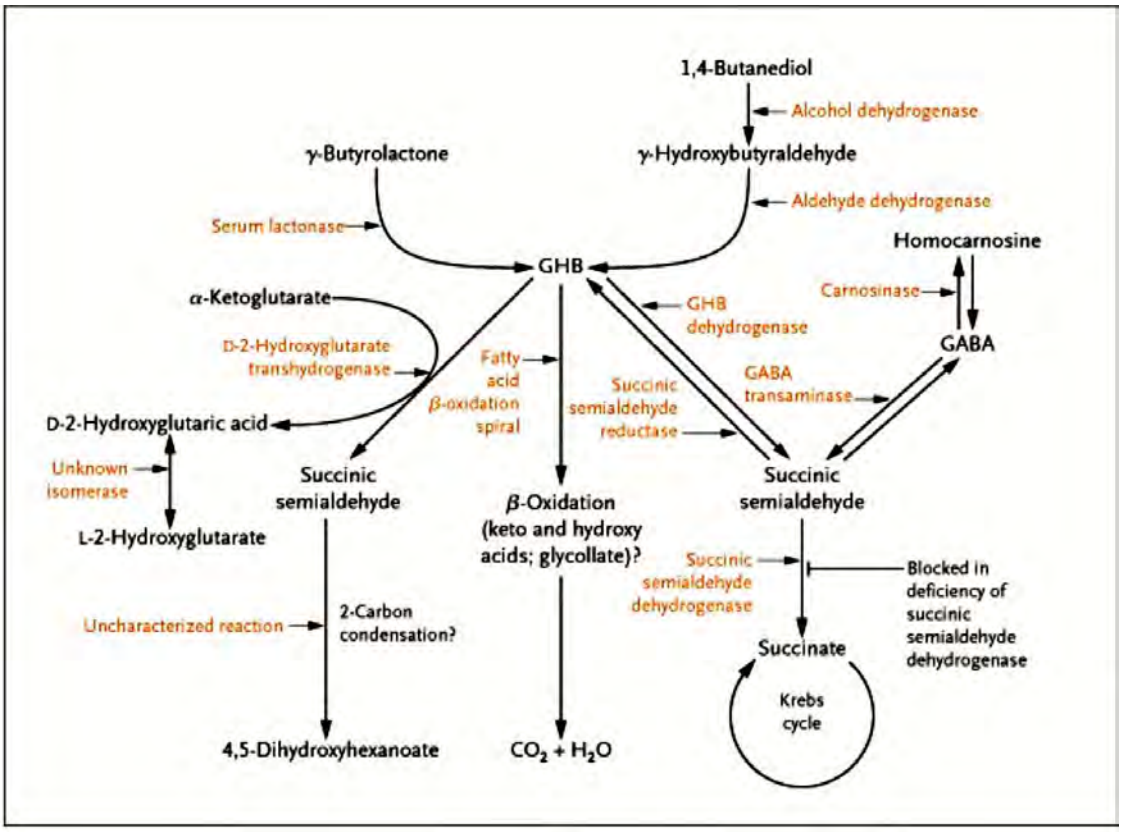

Figure 3. Proposed metabolism of GHB[212]. Three possible pathways for GHB degradation and the conversion to GABA are illustrated

GHB actions

Previous studies showed that GHB has a sleep-promoting effect, especially by inducing SWS and decreasing sleep latency and fragmentation, and these changes are GABAB mediated[118,216]. Since

GHB is a drug of abuse, the evaluation of its impact on dopaminergic system is important. Interestingly, it was shown that GHB effects on dopamine release are dose dependent: while at low doses GHB disinhibit dopamine neurons, enhancing dopamine release in different brain regions, at high doses it has hypodopaminergic effects.[217] Despite its increasing medical use, very little is known about the effects on arousal mediating nuclei. Recent animal data imply that GHB exerts its sleep and motor effects by inhibiting serotonergic neurons from dorsal raphe and cholinergic neurons from laterodorsal tegmentum, which might explain the hypotonia and NREM and REM sleep-promoting effects[218].

Neuroendocrine and protective effects of GHB

Early human studies, which preceded GHB use by body-builders, pointed to the stimulation of growth hormone and prolactine release[219,220,221]. Nevertheless, administration of GHB in rats and dogs failed to reproduce the effectiveness of GHB as a growth hormone secretagogue.[222] Interestingly, GHB was shown also to increase cortisol release after acute administration both in humans[223,224] and animals[225,226] but the mechanism is not identified.

Protective effects of GHB administration were tested on different tissues. For example, GHB reduced affected myocardial fibers after brain-induced (ischemia) myocardiac injury[227] or prevented the myocardial fibers death[228], limited histological and functional consequences of focal ischemic or excitotoxic insult of the brain[229-231], antagonized alloxan's diabetogenic actions[232], improved liver function and integrity after hypothermic preservation and increased the acceptable storage time of donor liver before transplantation[233,234].

6. Research outline, personal contribution and main results

a. Effects of age and gender on sleep and sleep EEG

Increased life expectancy as a result of reduced mortality, progresses in treating major age-related disorders, and increased quality of life, raises the need for healthcare strategies to tackle the complex problematic of aging. For this, an integrated approach is needed. Sleep is one of the elaborate neurological processes linking health, mood and cognition. The first part of my PhD thesis covers a subjective (questionnaires) and objective (polysomnography) approach to examine normal sleepers across age and gender. The major aims were to identify how sleep perception and sleepiness are affected by aging, to test if sleep architecture and EEG are dramatically affected as a result of aging *per se*, and to evaluate if effects of aging on sleep and sleep EEG are modulated by gender.

We found important interactions between age and gender affecting sleep. Women had better preserved sleep architecture and less sleep complaints, while their EEG spectral composition was more altered in low frequencies compared with men. Conversely, altered sleep structure in men was paralleled by increase in high frequency EEG. These results are of particular interest since normative sleep and EEG data for normal aging populations are spars. We concluded that sleep complaints in elderly must not be considered as merely a consequence of aging but should be investigated for sleep disorders or other underlying conditions.

b. Central and peripheral effects of gamma-hydroxybutyrate

The second part of my PhD thesis investigates the metabolic effects of gamma-hydroxybutyrate (GHB) in mice. Currently used to treat narcolepsy with cataplexy, non-hypnotic effects of GHB were reported more than 40 years ago, and recently became of interest in

narcolepsy patients. Moreover, the mode of action of this drug is still controversial. Previous work in our lab assessed receptors involved in hypnotic effects of GHB in mice and evaluated its effects on sleep in healthy volunteers. The increase in the growth hormone in humans, which might be the reason why GHB is abused by body-builders, the weight loss described as side effect in some of treated patients and decreased heat production suggest yet unexplored metabolic effects. In different experiment we have evaluated the effects of GHB on metabolism and central (cortex) and peripheral (liver) biochemical processes influenced by the drug administration in different mouse strains.

Acute administration of GHB increased corticosterone levels both in wild type and in GABAB ko mice, but growth hormone and prolactin were not significantly affected. Metabolomic profiling identified oxidation to succinic semialdehyde through hydroxyacid-oxoacid transhydrogenase as the major pathway activated by the drug. Interestingly, even at doses as high as 300mg/kg, GHB is not converted to GABA. Four days of GHB treatment resulted in decreased respiratory ratio independently of food intake, suggesting a shift in energy substrate from carbohydrates to lipids. Body composition evaluated by EchoMRI after 4 weeks of GHB administration showed a more balanced distribution of fat and lean mass in treated mice. Both C57BL/6J and GABAB ko treated mice showed slower weight gain, suggesting that weight change is not GABAB mediated. Fatty acids synthesis and omega-3 fatty acids were increased in the liver of the mice treated after 4 weeks. In the cortex, oxidized glutathione and s-adenosylhomocysteine were significantly reduced in treated mice while reduced glutathione was increased, suggesting possible anti-oxidant effects of GHB. In conclusion, GHB has important complex central and peripheral metabolic effects suggesting anti-oxidant and anti-obesity beneficial effects.

Personal contribution

For the human study, I helped preparing participants for polysomnographic recording, calculated questionnaires outcome, transformed polysomnographic recordings in EDF format, prepared recordings for spectral analysis, performed spectral analysis and analyzed all the data with the help of my supervisor Prof. Mehdi Tafti. I wrote the manuscript together with my supervisor and with inputs from the co-authors, and I created the figures and the tables. This work is ready for submission.

For the metabolic project, I participated in the design of the experiment with the help of my supervisor. I organized the administration of the drug with the help of Yann Emmeneger and Angelique Vaucher, performed the calorimetric and body composition experiments (with the help of Frederique Preitner), performed EchoMRI measurements, weighed mice and collected the blood, brain and liver (with the help from Yann Emmeneger, Angelique Vaucher and Sonia Jimenez), and analyzed all the data. The manuscript was written together with Julie Vienne and Mehdi Tafti, and was accepted for publication in the journal SLEEP.

Additionally, I have participated in several other projects. Amongst these, I collected clinical and laboratory findings in 1099 narcolepsy patients recruited by the European Narcolepsy Network, analyzed and wrote a paper as the first author (Luca G et al., J Sleep Res. 2013, 22:482-95; Annex I). This work is considered as a reference (first year citation: 15) in the largest ever narcolepsy with cataplexy population. I also participated in a study correlating subjective and objective sleep measures (similar to the work presented here) with cardiovascular risk factors and metabolic syndrome (Haba-Rubio J et al., Sleep, 2014, in press; Annex II). This important study shows for the first time that normal variations in sleep contribute little if any to metabolic syndrome and cardiovascular risk.

7. Discussions and perspectives

7.1 Normal sleep across age and gender

Even if we still do not know the answer to the apparently simple question "Why do we sleep?" there is unquestionable evidence that sleep duration and quality are important determinants of health[235]. A recent report of Centers for Disease Control and Prevention calls for promoting increased awareness of sleep, a behavior as important as dietary habits, smoking cessation, and physical activity[236]. The impact of sleep duration and quality on inflammatory processes[237], weight control[238], diabetes[239 240], and cardiovascular disorders [241] has been studied, but further longitudinal studies to distinguish between effects of sleep *per se* and those of sleep disorders and other determinants are required. Also, associations between mood disorders and sleep have been described[242,243] suggesting that many of the conditions with important social and economic burden influence sleep or might be triggered by sleep disturbances. Examining sleep characteristics across age and gender contributes to a better understanding of sleep functions and reveals how even subtle alterations in its structure may impact daily life and health. Also, given the association between age and several neurologic conditions (Alzheimer's disease), it is of major interest to track sleep changes, which precede and/or accompany these disorders.

In this study we explored the effects of age and gender on subjective sleep evaluation, sleep structure and sleep EEG in normal sleepers from one of the largest European cohorts, HypnoLaus. We have analyzed how 2211 subjects (40-80 years old) without sleep complaints evaluate their sleep and time-of-day preference (chronotype). Benefiting from a large age range with balanced gender distribution we could confirm and extend previous findings demonstrating the morningness preference in aging populations, independent from gender as previousely reported [244]. Nevertheless, a recent study, where over 14 000 Brazilian volunteers completed the

same chronotype questionnaire (MEQ), reported that men became more morning-oriented than women[245]. Even if in our sample no gender differences were identified with MEQ scores, they were present in sleep timing extracted from Pittsburgh Sleep Quality Index (PSQI). Used as an estimate of daytime quality and altered sleep, PSQI surprisingly revealed improved sleep quality and better functioning during the day with aging in subjects without sleep complaints.

Excessive daytime sleepiness in older subjects is associated with a number of factors, such as the presence of obstructive sleep apnea, pain, respiratory pathology, medication with sleepiness as side-effects, increased REM duration, male gender and increased PSQI score,[246] indicating that sleepiness in aging population might not result from age-related changes in normal sleep. The dissociation between age *per se* and sleep complaints was also previously suggested[247]. One of the important predictors of reporting sleep complaints is ethnicity[248] suggesting the need for future research on genetic contributions. For the majority of sleep components evaluated by PSQI genetic influences were also identified[249]. More recently it was shown that heritability of PSQI components (except sleep medication) ranged between 0.15 to 0.31 and contributed to approximately one-third of the variability of global subjective sleep quality[250]. Our PSQI results together with genome-wide SNP markers are currently being analyzed by an international consortium.

We showed that in the absence of sleep disorders, medication or alcohol, sleep architecture is not dramatically affected by normal aging. Since the observed changes in sleep structure are progressive in both genders, there is probably a gradual adjustment to these changes, ultimately leading to even improved self-assessed sleep quality with aging.

Sleep architecture and sleep EEG were shown to be under genetic control. Twin studies showed that temporal sequence of sleep stages across the night is completely concordant in monozygotic twins[251]. Homozygosity for the longer *PER3* allele (*PER3(5/5)*) has a considerable

effect on sleep structure, including SWS[156]. *PER3* polymorphism was also associated with higher homeostatic sleep pressure[252] conferring vulnerability to sleep loss and circadian misalignment[253]. Moreover, repeated polysomnography revealed significant stability in sleep architecture between nights, reflecting a trait-like signature[254]. Different genes, like *ADA, BDNF,* and *COMT* were associated with EEG sleep characteristics. For example, *ADA* (adenosine deaminase, which catalyzes the irreversible degradation of adenosine to inosine and contributes to the regulation of extracellular adenosine levels) polymorphism affects waking quality, the amount of SWS and delta power both in NREM and REM sleep[255]. Val66Met polymorphism of *BDNF* (brain-derived neurotrophic factor) affects theta EEG frequency and also response to citalopram treatment in patients with depressive disorders[256]. Val158Met polymorphism of *COMT* (catechol-O-methyltransferase) predicted interindividual differences in brain alpha oscillations in wakefulness and 11–13 Hz EEG activity in wakefulness, rapid-eye movement (REM) and non-REM sleep[257]. In addition, it was demonstrated that *COMT* polymorphism affects the response to chronic sleep deprivation[258], narcolepsy symptoms and the response to modafinil[101]. Given these associations, a genetic approach in our sample might explain interindividual variability and how these polymorphisms impact age effects on sleep.

Due to the cross-sectional nature of our study, some questions may be addressed:

- *What is the natural course of sleep in our selected normal population with age? Is there a role for gender?* In order to answer these questions a longitudinal reevaluation of this cohort will uncover insights about the evolution of normal sleep architecture and sleep EEG (the follow-up study will start soon).

- *What is the impact of genetic variations on our findings?* HypnoLaus cohort participants had undergone comprehensive genetic, somatic, and psychiatric investigations, which allow beside the characterization of sleep phenotypes and of sleep quality a GWAS approach to reveal candidate genes for sleep and sleep EEG parameters (ongoing work).

7.2 Metabolic effects of GHB

GHB is a "neurotransmitter" whose actions remain incompletely understood. If the effects of GHB on sleep were studied in narcolepsy patients, healthy volunteers and some mammals[121,122,216,259], its influence on metabolism started to be explored only recently in narcolepsy treated patients, mainly because of weight loss[121,122]. Potential metabolic effects were suggested by early observations, as changes in selected brain metabolites[206,226,260] and more recently, the stimulation of growth hormone[222,223], cortisol[261] and prolactin[262]. Moreover, weight loss reported by treated patients[263,264] strengthens the presence of important non-sleep related effects. One major difficulty in studying the effects of GHB is its variable actions at different doses and possible species-specific effects[222].

Our main interest in this project was to evaluate the central versus peripheral metabolic changes induced by GHB in different mouse strains (C57BL/6J, leptin deficient ob/ob, GABAB ko mice) after acute, short term, and chronic administration. For this aim, blood analysis, calorimetric evaluation, echoMRI and metabolomic approaches revealed unexpected significant metabolic changes

Increased in corticosterone after acute GHB administration is consistent between published studies[223,265], and this effect was considered to be centrally mediated, but GABAB independent[266]. This important finding needs further investigations due to the major metabolic

impact of corticosterone as well as the possible interaction with development of depression in some (predisposed) GHB-treated patients.

GHB effects on growth hormone in humans and mice raised debates about possible GABAA or other neurotransmitter receptors' involvement. For example, GHB promoted growth hormone increase in Parkinson patients, while sodium valproate and baclofen (GABAB agonist) had no effects.[221] The suppression of GHB-induced growth hormone release by pirenzepine, a muscarinic receptor antagonist, suggested a possible cholinergic involvement.[267] Nevertheless, in our experiments, the growth hormone secretagogue effect was not observed 1 hour after administration of 300 mg/kg GHB in mice, and similar results were also reported in rats and dogs[222]. Weight loss after GHB administration was reported in narcolepsy patients [268] but the mechanisms is not understood. It was proposed that weight loss is a result of improved sleep architecture, reduced narcolepsy specific symptoms and increased daytime activity[268]. However, in several cases the weight loss amounts up to 10-20 kg that cannot be explained by an increase in daytime activity, which is not actually documented. Also, in our study GABAB ko mice insensitive to GHB in terms of changes in sleep or motor activity presented a decrease in weight, suggesting that this effect is not centrally mediated, at least through GABAB pathway.

Acute and chronic administration of GHB resulted in increased succinate, metabolized by hydroxyacid-oxoacid transhydrogenase. An oxidation fate of GHB was described also by *in vitro* studies in the rats' liver[269]. Identification of the gene encoding for hydroxyacid-oxoacid transhydrogenase (ADHFE1) on chromosome 8q 13.1[270] opens new perspective in studying GHB metabolism. A dysregulation of this gene in ob/ob adipocytes and kidney suggests that the putative metabolic pathways wherein Adhfe1 functions are among those that are altered in obesity[271].

Metabolomics profiling after acute administration of GHB confirmed the increase in corticosterone level. Simultaneous serum measures are needed to link the systemic increase of corticosterone to changes in lipids and biliary acids. Interestingly, chronic administration of GHB revealed a few potential beneficial effects of the drug. Reductions found in glutamate levels, s-adenosylhomocysteine, and of the oxidative stress confirm previously reported GHB protective properties in the brain, cardiomyocytes, and pancreas[227,228,232,272].

Our findings raise several questions and new hypotheses, which may be addressed in future projects:

- *What are the differences in the pathways affected by GHB administration in narcolepsy patients?* The same protocol may be used to assess GHB metabolic effects in mouse models of narcolepsy. Also, similarities and differences between these results and the metabolomic profiles in GHB-treated narcolepsy patients (ongoing in our lab) might help our understanding of the causes of weight loss in these patients and eventually reveal new pathways contributing to cataplexy mechanisms.

- *Which are the metabolic effects independent of corticosterone increase?* Adrenalectomized mouse models can be used to answer this question.

- Since sleep and locomotion effects of GHB are conditioned by the presence of functional GABAB receptors, the metabolic effects of GHB seem independent of central GABAergic mechanisms. *Whether pharmacologic pre-treatment with cholinergic/serotoninergic/dopaminergic inhibitors alter somatotropic response in mice needs further work.*

- *Adhfe1* gene might play an important role in the metabolism of GHB. *Studying how GHB alters the expression of Adhfe1 in different tissues and the effects of GHB in mice lacking Adhfe1 will allow further understanding of GHB metabolic effects.*

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Age and gender-related sleep changes in the general normal population

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Abstract

The effects of age and gender on sleep are increasingly recognized. However, available studies either compared young to middle age/old subjects or included populations with mixed normal and pathological sleep. Here, we re-evaluated these effects in normal sleepers from a large representative sample of the general population: the HypnoLaus cohort. Subjective evaluation of sleep was assessed by questionnaires in 5064 subjects, among whom 2211 were considered as free of any sleep disorder. Objective evaluation was performed by polysomnography in 2160 subjects and 890 were considered normal sleepers. We confirm that the old age is strongly associated with morning chronotype. This chronotype is also strongly associated with a better daytime functioning independent of age and gender. Surprisingly, aged normal sleepers, especially older women, complained less about sleepiness during the day and even pathological sleepiness (Epworth Sleepiness Score > 10) was significantly lower than younger subjects. Also, self-reported sleep quality and daytime functioning improved with aging. Although women slept (objectively) slightly more than men, total sleep time was not affected by age. Sleep latency increased with age in women only while sleep efficiency decreased with age in both genders, especially in women. Deep slow wave sleep also decreased with age but men were more affected. Spectral power densities within slow waves (< 5Hz) and fast spindle (14-14.75Hz) frequencies decreased while theta-alpha (5-1Hz) and beta (16.75-25Hz) power densities in non-rapid eye movement (NREM) sleep increased with aging. In REM sleep, aging was associated with a progressive decrease in delta (1.25-4.5Hz) and increase in higher frequencies but beta frequencies were not affected in women. Our study constitutes normative data on natural history of sleep across age and gender and can be used as standards to evaluate changes induced by pathological aging. Our findings indicate that the presence of sleep complaints should not be viewed as part of normal aging, but should prompt the identification of underlying comorbidities.

Introduction

As many other biological functions, sleep is altered with aging. The exact contribution of aging *per se* or of the interaction between age and multiple factors (e.g., biological, socio-economical, psychological) to sleep changes is difficult to disentangle. Sleep structure, duration and quality have different dynamics during aging, and common patterns can be identified both in healthy aging and in subjects with intrinsic sleep disorders. These include increased sleep onset latency and wake after sleep onset, increased non-rapid eye movement (NREM) sleep stage 1 (shallow sleep) and reduced amount of slow wave sleep (SWS or deep sleep)[1-3]. Objectively measured sleep duration, by polysomnography, was found to be shorter in the elderly in comparison to young and middle-aged adults[4], but when comparing subjects aged 60 with subjects over 70 years this trend was not observed, suggesting that the decline is not linear till old age[5].

Beyond sleep architecture, normal aging affects also specific electroencephalographic (EEG) features. For example, not only the amount of slow wave sleep was reported to decrease, but aging was also associated with diminished power density within the slow frequency range (0.5-4.5 Hz) during NREM sleep, the so-called delta power, a highly reliable index of sleep intensity[6,7]. While many studies agree about the reduction in delta power with aging, there are also reports indicating a significant interindividual variability in delta power in elderly.[8] With aging, the beta power density, that measures high frequency EEG activity, was shown to increase, along with decreases in sigma, delta and theta band powers in almost all sleep stages.[4]

An important factor affecting the modulation of sleep with aging is gender. Previous studies reported poorer subjective sleep quality in aging women[9], but polysomnographic data showed a better sleep structure[10] in terms of increased SWS and decreased wakefulness after sleep onset[11] compared to aging men. It was proposed that different psychosocial factors in men and women may contribute to dissimilarities in the perception and evaluation of symptoms[11] which may contribute to the differences in self-rating sleep quality. Women usually complain about difficulties in falling asleep[12] and this is consistent with increased prevalence of insomnia with aging in women[13], while men have more problems in sleep maintenance, lighter sleep and more frequent sleep related breathing disorders[14]. Gender differences in sleep were mostly attributed to hormones, but social and cultural factors might also participate[15]. Other studies offer contradictory gender differences, with men complaining more about their sleep[16].

More recent studies revealed gender differences in the sleep EEG. If some studies concluded that aging in men and women are similar[4], others showed that disentangling the effect of gender on EEG spectral composition might be complicated because of methodological issues related to spectral analysis data processing[1]. Differences between men and women were suggested in terms of brain maturation, perceived sleep, NREM-REM distribution, or EEG features[17-24].

A major source of sleep alteration in aging populations is the increasing occurrence of sleep disorders. Insomnia is one of the most common sleep disorders with a major impact on daytime functioning, alertness, medical status,[25] sleepiness and cognitive functions. Its prevalence and severity increases with aging, especially in women.[26] The prevalence of obstructive sleep apnea syndrome varies between 4-7% in men, and between 2-5% in women[27]. Restless legs syndrome (RLS) affects between 4 and 29% of the general population[28-30] and is associated with depressive symptoms, decreased sleep quality[31] and reduced quality of life[32]. Given the high prevalence of sleep disorders in older subjects with their well-documented impact on sleep structure, careful exclusion of those subjects is necessary to evaluate the natural history of sleep changes across age and the effect of gender.

Thus, the aim of this study was to perform a detailed characterization of sleep in relation with age and gender in one of the largest European sleep cohorts: the HypnoLaus study. Moreover, the impact of changes in normal sleep on daytime sleepiness, perceived sleep quality and daytime functioning, as well as their interactions were also analyzed.

Methods and participants

1. Participants' selection

HypnoLaus Sleep Cohort Study participants were recruited from the original CoLaus sample during the first follow-up. The CoLaus study was designed to assess the prevalence of cardiovascular and metabolic disorders, their risk factors and to identify their genetic determinants[33]. All subjects (6733 participants, aged 35-75 years), randomly selected between 2003 and 2006 from the adult general population of Lausanne-Switzerland, underwent a comprehensive somatic and genetic investigation. HypnoLaus participants completed a series of self-rating sleep pattern and disorder questionnaires. 5064 participants, who accepted to

participate in the follow-up, correctly filled in the sleep questionnaires (46.5% women). There were no demographic differences between original CoLaus cohort and HypnoLaus sub-population. Out of these, 3051 consecutive subjects were invited for an ambulatory full-night polysomnography, without any prior selection based on the questionnaires. 2168 subjects (71.1% of contacted) accepted to participate. Those who declined were 8.2 years older, sleepier, and BMI and PSQI scores were slightly higher (+0.4kg/m^2 and +0.3 points, respectively). In 60 recordings technical issues were encountered but 54 of these subjects accepted to repeat the study, resulting in a final population of 2162 subjects (51.2% women).

2. Sleep questionnaires

All participants were asked to fill in questionnaires to assess sleep quality, sleepiness, and chronotype. Chronotype was assessed by the Horne and Ostberg Morningness-Eveningness Questionnaire (MEQ), which is based on 19 questions. The chronotype classification of subjects was performed according to Taillard et al.,[34]. Epworth Sleepiness Scale is widely used to asses sleepiness in the general population and in different sleep disorders, and rates the chance of falling asleep in eight different situations.[35] Scores higher than 10 (range 0-24) are associated with excessive daytime sleepiness. The Pittsburgh Sleep Quality Index (PSQI) is an effective instrument for subjective evaluation of sleep quality over seven domains[36]: subjective sleep quality, sleep latency, sleep duration, sleep efficiency, sleep disturbances, use of sleep medication, and daytime dysfunctions over the last month. For each domain, the score ranges between 0 and 3, where 3 is the negative extreme. A total score higher than 5 (out of 21) is associated with poor sleep quality[36]. There was a 6 to 18 month delay between the questionnaire administration and the polysomnographic evaluation. Therefore, in the morning following the polysomnographic recording participants completed another questionnaire providing information about the quality of their sleep in the previous night, the amount of alcohol consumed 4 hours before going to bed, and their current medication.

3. Polysomnography

Full ambulatory polysomnography was performed using a digital portable sleep/wake recording system (EMBLA Titanium®, Embla systems, Inc, Broomfield, CO, USA). Sensors were placed by a trained technician at CIRS (Center for Investigation and Research in Sleep,

Lausanne University Hospital, Lausanne). The following measurements were performed: electroencephalogram (EEG) from frontal, central and occipital areas (F3-M2, F4-M1, C3-M2, C4-M1, O1-M2, O2-M1) according to the international 10/20 electrodes configuration system, right and left electro-oculogram (EOG), submental electromyogram (EMG), right and left leg EMG, thoracic and abdominal breathing movements (inductance plethismography), electrocardiography, pulse oxymetry, respiratory airflow via nasal cannula connected to a pressure transducer, and body position.

Sleep stages, leg movements and arousals were scored according to the 2007 AASM criteria[37]. Apneas/hypopneas were scored according to the AASM 2013 rules[38]. The average number of apneas/hypopneas per hour of sleep (apnea-hypopnea index [AHI]) was calculated. Analyzed PSG variables were: sleep duration or total sleep time (TST): total minutes of any stage of sleep from sleep onset to morning awakening; wake after sleep onset (in minutes); percentage of stage 1, stage 2, SWS, and REM sleep: percentage of TST spent in each sleep stage; sleep efficiency: ratio between the total sleep time and time spent in bed; arousal index (AI), periodic leg movements index during sleep (PLMSI), 4% oxygen desaturation index (ODI): number of events (AI, PLMSI, and ODI, respectively) divided by hours of TST.

4. Spectral analysis

Signals were filtered by a high-pass filter (EEG and EOG: −3 dB at 0.5 Hz; EMG: 10 Hz), a low-pass filter (EEG: −3 dB at 35 Hz, EMG: 70 Hz), and a notch filter at 50 Hz. Data were sampled at 256 Hz. C3-M2 EEG derivation was subjected to spectral analysis. Sleep stages and the events were visually scored by two registered PSG technologist on a 30-s epoch basis (Somnologica® Software, Medcare Embla® Flaga, Reykjavik, Iceland) according to AASM criteria[37]. The EEG power spectra of consecutive 30-s epochs (average of 4-s epoch interval with 50% overlap, fast Fourier transform routine, Hamming window, frequency resolution 0.25 Hz) for NREM sleep (stage S2 and stage S3) and REM sleep were calculated using PRANA® software[39]. Movement, arousals and respiratory events were considered artifacts and removed from the analysis. An automatic artifact detection and removal was performed using PRANA® software[39], but each recording was also visually inspected for the correction of the artifact selection and additional artifacts were manually marked. All epochs containing artifact were removed from spectral analysis. NREM-REM cycles were defined according to Feinberg and

Floyd[40]. Sleep-onset REM period was defined as at least one epoch of REM sleep occurring within the first 15 min of sleep. NREM and REM spectral analysis was separately calculated for the first four sleep cycles. Besides EEG spectral composition, delta (0.75-4.5Hz), theta (4.75-8 Hz), alpha (8.25-11 Hz), slow spindles (11.25-13 Hz), fast spindles (13.25-15 Hz) and beta (15-25 Hz) bands were calculated. To account for interindividual differences, EEG power spectra were normalized by dividing the power in each 0.25 Hz bin by the total power for NREM and REM sleep over 0.75-30 Hz frequency range.

5. Selection of subjects without sleep complaints

To assess the effects of age and gender on subjective measures a "non-complaining" population was selected based on information obtained from PSQI. Subjects who declared sleep complaints (mainly environmental), shift workers, those taking any sleep medication, experiencing pain during night (more than twice per week), sleep related breathing problems, and those with estimated sleep efficiency less than 85% and increased sleep latency (>30 minutes) or frequent awakenings during the night or early in the morning (more than twice per week) were considered "not-normal".

For the polysomnographic data the normal population was defined based on the absence of medication which interferes with sleep structure and/or duration, less than 2 glasses of wine (or equivalent) consumed 4 hours prior to the sleep recording, sleep duration >5 hours, sleep efficiency >80%, absence of sleep onset REM periods, 4% oxygen desaturation index and periodic leg movement index less than 15/hour. Available criteria indicate >85% sleep efficiency as "normal". Since in older populations this criterion reduces significantly the number of included subjects, we compared those with sleep efficiency between 80-85% with the rest of the sample, and no significant differences were found in terms of gender, BMI and sleep architecture.

6. Statistical analysis

Subjective parameters analyzed were: MEQ scores and chronotype, ESS score, PSQI total score and, separately, each domain and sleep estimates (bedtime, sleep onset latency, wake up time, total sleep duration). Subjective habitual sleep duration was used as continuous variable or as categorical (less than 6h, between 6-7.5h and more than 7.5h of sleep). The following

polysomnographic data were used: total sleep time, sleep onset latency, wake after sleep onset, sleep efficiency, arousal index and sleep stages distribution, periodic leg movement and respiratory events during sleep scored according to. Another factor analyzed was body mass index (BMI) (used in analyses as continuous or categorical variable, with subjects classified as "normal" (if BMI<25kg/m^2), overweight (25≤BMI<30kg/m^2) or obese (BMI ≥30 kg/m^2).

To analyze the effects of age, four 10-year age groups were created (40-49y; 50-59y; 60-69y; 70-80y) and one-way ANOVA/Kruskal Wallis Test or χ^2 were used to test the effect of age (as categorical variable). T-tests or Fisher's exact tests were performed to test the effect of gender. To assess the interaction between age and gender for questionnaires, polysomnographic and spectral analysis data, two-way ANOVA (followed by post-hoc multiple comparisons tests) was performed. To derive the mid-sleep point, bedtime, sleep onset, wake-up time sleep estimates from PSQI were used. Multinomial logistic regression was used to evaluate the chronotype in relationship with age (unadjusted model) or in relationship with daytime functioning (adjusted for age and gender), and the association between BMI and subjective sleep duration (adjusted for age and gender). Partial correlation analysis was used for testing the association between demographic, subjective, and objective data.

Results

Subjective sleep evaluation

After removing subjects with declared disturbed sleep, 2211 participants (51.3% women; mean age 56.57±10.25 years) were considered "non-complaining" and included in the analysis. Population characteristics and self-rated sleep pattern results are summarized in Table 1.

Chronotype

Aging was associated with a gradual shift towards morningness. The risk of being morning type was 7.62 times higher in older subjects compared with younger ones (Table 2). The diurnal preference measured by the MEQ could also be approximated by mid-sleep point, time to go to sleep, time to fall asleep and time to wake up from the PSQI (r=-0.537, r=-0.483, r=-0.485 and r =-0.444, p<0.001 respectively, partial correlation, adjusted for age and gender). Daytime functioning was associated with chronotype: morning type subjects had higher chance to report a

better daytime functioning compared with evening type subjects, independent of age and gender (unadjusted model: odds ratio [OR] 6.63, 95% confidence interval [95%CI] 1.32-33.21, $p<0.001$; adjusted model for age and gender: OR 5.61, 95%CI 1.11-28.48, $p<0.001$). By aging subjects went to bed and fell asleep earlier and their sleep duration was longer. Gender effect was stronger for younger subjects, with women going to bed and falling asleep earlier, and sleeping longer. It was expected that mid-sleep point advances by age, but due to the increase in sleep duration, the mid-sleep point was shifted towards morning (Table 1).

Daytime sleepiness

The ESS score decreased with aging, and the reduction was larger in women (two-way-ANOVA for "age", "gender", "age x gender", $p<0.001$, Holm-Sidak correction for multiple testing). Also, the prevalence of pathological sleepiness, evaluated by an ESS higher than 10 diminished with aging. If in subjects aged between 40-60 years the prevalence was around 12%, in the very old ones it decreased to 5.4% in men and 2.8% in women (Table 1). The only self-assessed sleep variable correlated with increased daytime sleepiness was short sleep duration. Subjects who slept less than 6h/night had the highest ESS scores, compared with normal/long sleepers (two-way ANOVA, $p < 0.001$ for "age" and "total sleep time category", $p = 0.77$ for the interaction).

Subjective sleep quality

Self-reported sleep quality and daytime functioning measured by PSQI improved with aging (Table 1). Besides age and gender, subjective sleep duration was correlated with BMI: the "best" duration for a normal BMI was between 6 and 7.5h and short sleep duration (less than 6h) was associated with increased BMI, compared with long sleep duration (multinomial logistic regression, for BMI>30kg/m^2 OR: 4.62, 95%CI 2.26 to 9.36, $p<0.001$, adjusted for age and gender).

Polysomnographic data

890 subjects out of 2160 subjects who underwent polysomnographic recording (PSG) were considered normal sleepers. Women slept on average 19 min longer than men, but overall, sleep duration was not affected by age. Sleep latency increased with age in women only (two-

way ANOVA for age p=ns, gender p=0.009 and their interaction p=0.4). Sleep efficiency decreased with age especially in women (two-way ANOVA for age $p<0.0001$, gender p=0.02, interaction p=0.11). SWS amount decreased with age independent of age and gender (but men were more affected). REM sleep duration was inversely correlated with REM sleep latency (r=-0.282, $p<0.0001$), and the same negative correlation was found between SWS latency and SWS amount (r=-0.303, $p<0.0001$). As expected, higher proportion of S1 was associated with decreased sleep efficiency (r=-0.258, $p<0.0001$) and increased sleep fragmentation (r=0.319, $p<0.0001$). Men, who spent more time in S1, independent of age, had less SWS and REM sleep (Table 3).

To summarize, sleep onset latency, stage 1 and SWS amounts were gender dependent, irrespective of age, sleep duration, and BMI, while sleep efficiency, number of awakenings and wake after sleep onset were age dependent, irrespective of gender and BMI ($p < 0.003$, general linear model with factors "age group", "gender", "age group x gender", corrected for sleep duration and BMI).

Subjective versus objective sleep evaluation

The best estimators of sleep duration were subjects older than 70 years, while younger ones underestimated their sleep amount (Spearman correlation between declared sleep in PSQI and PSG recorded sleep: r=0.13, p=0.012 for the subjects aged 40-49 years and r=0.451, p=0.003 for those aged 70-80 years). SWS and REM sleep amounts were not associated with any of the sleepiness or sleep quality measures. The relationship described between BMI and declared sleep duration was not significant when taking into account the total sleep time recorded during polysomnography.

Spectral analysis

Out of 890 PSG recordings of normal sleepers, 776 recordings were subjected to spectral analysis. 114 recordings were not used in the analysis due to: lack of signal on C3-M2 EEG channel, mirror of ECG in EEG channels, increased amount of artifacts which increased the probability that remaining amount to be non-representative for the whole night EEG. There were no age or gender differences between excluded subjects and remaining ones.

NREM sleep

In Fig. 1(left) are represented the average relative NREM EEG spectra for each age group. A significant decrease with increasing age was observed in low frequencies (1-3 Hz; 4.25-5.00 Hz) and spindles (14.00-14.75 Hz) and an increase in theta-alpha (5.00-11.00 Hz), and beta frequencies (16.75-25 Hz) (two-way ANOVA, "age group", "bin" and "age group x bin", $p<0.001$). When analyzing the association of different sleep stages with spectral results, men and women were differently affected. For example, increased amount of stage 1 in older women was negatively associated with spindles frequency power ($r=-0.393$, $p=0.02$), while in men, no association was found. This finding suggests important gender differences in spectral EEG composition and its relationship with sleep architecture. We therefore tested first the effect of gender overall, and then assessed the effect of age separately for women and men. When considering the absolute powers of spectral analysis, women had higher power in a large frequency range (0.75-1.25 Hz; 4.75-13 Hz, $p<0.001$) compared with men, but after normalizing for total power, women had lower power for a small frequency range in the delta (2-2.75 Hz) and beta band (16.25-25 Hz) and higher power in theta-alpha band (5.75-10.25 Hz) ($p<0.001$, t-test) (Fig 1, right). With aging, women showed an increasing trend in power density in high frequencies (Fig. 2, left), except fast spindles. In men (Fig. 2, right) the largest age-related increase was found in the beta band.

REM sleep

Spectral composition of REM sleep was not affected by sleep structure, duration, or efficiency. Nevertheless, higher index of spontaneous arousal was associated with higher alpha power ($r=0.112$, $p=0.001$), and total arousal index was correlated with power in slow spindles frequency band ($r=0.136$, $p=0.0003$, partial correlation adjusted for age and gender).

Except very low frequencies (0.75-1.25 Hz), which were not altered, aging was associated with a progressive decrease in delta power and an increase in power in high frequencies (Fig. 3, left). The interaction between age and gender was also evident in REM sleep. Women had higher theta and lower beta frequency powers, while men had higher power in low frequency range (Fig. 3, right). To understand how age affects specifically each gender, age groups were compared separately in men and women. For women the only decrease was observed in delta frequency

range, while alpha and spindles power increased. Importantly, very high frequencies (high beta) were unchanged in women with aging. On the contrary, the most important increase in men was found in beta frequency range (Fig. 4), followed by increase in spindles band. Delta frequency in REM sleep was not affected in men with aging, yet theta power decreased.

Sleep cycles

To answer the question if the previous described findings are influenced by the time course of the night sleep, EEG spectra were compared between each sleep cycle. Different EEG frequency bands were analyzed for each sleep cycle. During NREM sleep an important age effect was observed in alpha, theta and delta bands in women (Fig. 5). Women aged between 70-80 years had the largest reduction in delta and the highest increase in alpha and theta power density. Age effect in men was observed only for the last sleep cycle within delta and alpha bands (Fig. 5).

Discussions

The present work aimed to reassess sleep, with special focus on its natural history across age and gender in a large representative sample of normal sleepers from the general population of Lausanne. To that end, subjective and objective measurements of sleep and sleep habits were analyzed.

Chronotype

Our results confirm that old age is associated with higher prevalence of morning types, when using MEQ.[41, 42-45] Both circadian clock and environmental factors regulate the chronotype. The difference between morning and evening types in terms of daytime functioning and total PSQI score was also described and explained previously by "social jetlag" experienced on a daily basis by evening-type subjects.[46] Dissipation of gender differences due to aging is also supported by previous work[45]. Shifting to morningness explains the earlier time in bed while later time to wake up and longer sleep duration with aging might contradict the circadian phase advance hypothesis in older subjects. Recent data suggested that sleep duration seems independent of chronotype[47-50]. One important environmental factor might be the retirement, which would

attenuate the difference between work and free days (social jetlag). The exact time of retirement was not available in our population.

Daytime sleepiness

Surprisingly, by aging people declare themselves less sleepy. Young age was previously associated with increased sleepiness[51,52] but it was shown that, at very old age, people experience again increased sleepiness[52], which is not supported by our results. Also, in our study, the lowest ESS scores were obtained in subjects over 70 years of age. Taking into account the variability in reported prevalence of sleepiness in general population, the one identified in our study is lower than previously reported[53-56] suggesting that reported sleepiness is not related to normal variations in night sleep, but is a consequence of sleep disorders or other age-related comorbidities, which impact daytime alertness.

Subjective sleep quality

Declared sleep quality improved with age, as well as the daytime functioning. Similar results were reported in other cohorts[57 58]. Increased reports of pain (as a result of accumulation of chronic disorders) and the use of sedatives can contribute to subjective sleep alterations[59]. For this reason, we considered only those subjects who were free of medications affecting sleep and those who did not complain about pain. One possible explanation for better rating of daytime and sleep quality is an adaptation of expectations about sleep in older populations[16] or an acclimatization to sleep changes over time[60]. Recent data[58] suggest an association between self-rated sleep quality and duration in aging populations with markers of cellular aging, as telomere length, which reinforce the importance of promoting and maintaining healthy sleep in aging.

Effects of age and gender on sleep structure

Sleep structure is strongly influenced by age and gender. These findings are supported by previous studies, especially the fact that men have more shallow sleep than women[2,11,61]. Alterations in sleep structure with aging, especially the decrease in SWS amount could be attributed to hormonal influences, such as a decrease in growth hormone pulse observed both in men and women[62,63]. Sleep architecture is more affected in men. Age-related differences in sleep stage amounts are more pronounced after 60 years of age, and this may be one of the reasons why

other studies, which analyzed sleep in young and middle age adults[64], did not find differences in REM sleep amount. The decrease in SWS starts earlier, in the middle age, both in men and women and was well-documented previously[3,24,65,66]. Interestingly, despite the fact that SWS is an index of sleep quality and intensity, aged subjects are less sleepy than younger ones even if they lose most of their SWS[67]. Experimental disruption of SWS in elderly resulted in increased sleepiness[67], but the daytime functioning was not impaired[68], suggesting that at this age subjects manage better their sleepiness. A meta-analysis of sleep studies published between 1978 and 1983 identified gender as one of the most frequent variable affecting sleep structure and sleep disturbances, and despite the fact that both genders experience increased sleep alteration with age, older women reported the changes more accurately[11].

An interesting factor, possibly affected by sleep duration and sleep disturbances, is increased BMI. In terms of subjective data, higher BMI was associated with both long and short sleep duration, but the strongest effect was found in very old men with short sleep. The same significant association between increased BMI and short sleep duration was reported by several other studies[69-71] but in most population studies the presence of sleep disorders were not taken into account. Nevertheless, a lack of association between sleep duration and BMI in subjects without sleep disturbances was also described,[72] implying that overweight/obese subjects might subjectively underestimate their sleep duration or they present yet undetected sleep disturbances. Note that we found no association with objectively measured total sleep time and BMI.

Effects of age and gender on EEG spectral composition

Age-related changes in the sleep EEG spectral composition, as the progressive power density decrease in low frequencies accompanying the reduction in SWS amount are concordant with previous results[64,73]. Important gender differences prompted us to test the effect of age separately in men and women. Another major component of aging, accentuated by gender, was the spindles frequency spectrum. Our findings showing an increase in slow spindle frequency and attenuation in fast spindle frequency band confirm the hypothesis of two distinct components[64], differently affected by the aging process. EEG and brain imaging studies demonstrated different origins[74] and distinct temporal dynamics[75] of spindles, which might be differentially affected by aging. If delta and spindles activity during NREM sleep were extensively studied because they

share a mutual temporal relationship, the same thalamocortical mechanism, and similar homeostatic regulation,[76] the role of theta-alpha bands is less examined.

In spite of a better preservation of sleep architecture with aging in women, they had lower delta and beta power and higher alpha and sigma power densities than men. Previous studies which used the same principle for normalizing the data[77] reported also that, in spite of a higher absolute delta power, women had lower delta power contribution to the total power, while beta band was attenuated compared with men. The functional significance of these changes are unknown but might be of major importance in terms of gender differences in sleep need and associated daytime alertness and cognitive processes.

Fast EEG frequencies (>15 Hz) were previously associated with insomnia,[78] a sleep disorder with increased prevalence in women with aging, [79] but it was also suggested that in middle age subjects it is difficult to distinguish between age-related changes and insomnia[80]. In our study beta frequency was higher in men than in women, both in NREM and REM sleep. Age-related elevated EEG beta power found in NREM sleep in men may not be a marker of central arousal, since the same pattern was also found in REM sleep. Contrary to previous findings, which suggested a better preservation of delta power in NREM sleep in men and in REM in women,[77] in our sample women had lower relative delta power also in REM sleep, and age effect on this frequency band was more pronounced in women compared with men. Methodological issues (e.g. normalization of data, larger sample size) may explain some of the differences between previous findings and ours.

Limitations

We report in this study how age and gender contribute to the natural changes in sleep quality, structure, and EEG spectral composition in one of the largest population-based cohorts. The main contribution of age and gender on normal sleep evolution with age was assessed both subjectively and objectively, and the presence of sleep pathologies was carefully excluded. In spite of these strengths, our study has several limitations. One of the limitations (as in previous studies) is that sleep-related questionnaires were self-administrated. Although errors in filling in the answers or misunderstanding of the questions cannot be excluded, the strong correlation

found between MEQ and timing described in PSQI as an example, suggests that the rate of error was low. Also, there was a time gap between the administration of the questionnaires and polysomnography. However, it was shown that PSQI score remains stable over a year[81] and sleep quality estimated in the morning following the polysomnography in our study was strongly correlated with PSQI, with better sleep quality in those with lower PSQI scores. Finally, lack of information about napping during the day before polysomnography could impact our findings, especially our spectral analysis results. Finally, our study did not include subjects younger than 40 years (well-studied previously).

Conclusions

Age-related changes in sleep do not affect subjective sleepiness or daytime quality. Normative data for normal sleep should be adapted for aging populations to reduce overmedication in elderly. Presence of sleep complaints should not be viewed as part of normal aging, but should prompt the identification of underlying comorbidities. Moreover, aging men and women are affected differently by changes in sleep pattern, which start progressively, at around 40 years of age. In spite of better sleep structure and less sleep complaints, women's sleep seems to be more affected in terms of spectral profiles with so far unknown consequences. The presence of significantly higher beta power in men suggests that they are more vulnerable to sleepiness and shallow sleep, and changes in this frequency range might be more important than the decrease in delta power. How our findings relate to changes in brain structures generating low (delta), spindles, and high frequency (beta) oscillations in NREM and REM sleep or to frequent neurologic comorbidities (e.g., Alzheimer's disease) in aging populations needs a close follow up of our cohort (in progress).

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Table 1. Effects of age and gender on sleep habits.

	40-49.9y.o. (N=708)		50-59.9y.o. (N=688)		60-69.9y.o. (N=553)		70-80y.o. (N=262)	
	Women (N=362)	**Men (N=346)**	**Women (N=348)**	**Men (N=340)**	**Women (N=301)**	**Men (N=252)**	**Women (N=124)**	**Men (N=138)**
PSQI_category (% positive) **(2)**	20.6	18.8	18.9	18.1	11.1	11.0	10.4	7.4
Psqi_total score **(2)**	4.00 ± 1.9	4.04 ± 1.7	3.88 ± 1.8	3.84 ± 1.7	3.32 ± 0.1	3.44 ± 1.7	3.20 ± 1.5	3.01 ± 1.6
Component 1-sleep quality **(2)**	0.81 ± 0.6	0.82 ± 0.6	0.78 ± 0.6	0.77 ± 0.6	0.71 ± 0.5	0.63 ± 0.5	0.64 ± 0.6	0.55 ± 0.5
Component 2-sleep latency **(1)**	12.10 ± 8.4	11.70 ± 7.4	12.50 ± 8.2	11.20 ± 7.4	12.8 ± 8.2	12.10 ± 7.9	14.50 ± 8.5	10.9 ± 7.4
Component 3-sleep duration **(1,2)**	7.20 ± 0.9	6.67 ± 0.8	7.21 ± 0.8	6.93 ± 0.9	7.58 ± 0.9	7.43 ± 1.1	7.73 ± 0.9	7.67 ± 0.9
Component 4-sleep efficiency **(2)**	95.14 ± 5.1	95.11 ± 4.5	94.85 ± 5.3	95.12 ± 5.1	93.70 ± 5.3	94.06 ± 5.4	93.25 ± 5.5	93.19 ± 5.3
Component 5-sleep disturbances **(1,2)**	1.01 ± 0.4	0.94 ± 0.4	1.09 ± 0.4	0.93 ± 0.4	1.03 ± 0.4	1.02 ± 0.4	0.99 ± 0.4	1.06 ± 0.4
Component 6-sleep medication								
Component 7-daytime dysfunction **(2,3)**	0.67 ± 0.7	0.60 ± 0.6	0.54 ± 0.6	0.57 ± 0.7	0.36 ± 0.6	0.49 ± 0.7	0.46 ± 0.6	0.30 ± 0.5
Time to go to bed **(1,2)**	23:00 ± 0.9	23:15 ± 0.9	22:53 ± 0.7	23:05 ± 0.9	22:58 ± 0.9	23:05 ± 0.9	22:57 ± 0.9	23:03 ± 0.9
Time to fall asleep **(1,2)**	23:13 ± 0.9	23:28 ± 0.8	23:06 ± 0.8	23:17 ± 0.9	23:11 ± 0.9	23:17 ± 0.9	23:13 ± 0.9	23:14 ± 0.8
Time to wake-up **(1,2)**	6:45 ± 1.0	6:40 ± 0.9	6:40 ± 0.9	6:31 ± 1.1	7:15 ± 1.0	7:09 ± 1.1	7.29 ± 0.8	7:27 ± 0.8
Mid-sleep point **(2)**	2.59 ± 0.4	3:01 ± 0.4	2:53 ± 0.4	2:53 ± 0.4	3:13 ± 0.3	3:13 ± 0.4	3:21 ± 0.4	3:20 ± 0.4
Epworth Sleepiness Scale **(1,2,3)**	6.58 ± 3.7	6.11 ± 3.6	6.06 ± 3.7	6.28 ± 3.8	4.72 ± 3.2	5.46 ± 3.3	4.11 ± 3.1	5.02 ± 3.1
Epworth Sleepiness Scale _category (%positive) **(2)**	14.7	10.4	12.8	11.2	5.4	5.8	2.8	5.4
BMI **(1,2)**	24.60 ± 4.5	26.01 ± 3.4	24.92 ±4.5.	26.36 ± 4.0	25.98 ± 4.9	27.44 ± 4.4	26.50 ± 4.7	27.40 ± 4.1
Horne and Ostberg questionnaire **(2)**	57.20 ± 9.2	57.81 ± 8.1	59.29 ± 8.2	59.47 ± 8.5	60.82 ± 8.9	61.57 ± 8.4	62.08 ± 7.5	63.61 ± 7.1
Definite morning (%)	8.4	5.9	11.6	9.6	17.2	16.5	16.1	22.0
Moderate morning (%)	13.4	14.8	16.5	20.7	19.5	24.3	30.1	27.1
Neither (%)	50.0	53.1	51.4	50.6	45.7	44.3	44.1	44.1
Moderate evening (%)	14.0	18.2	13.5	11.1	12.4	9.1	6.5	5.9
Definite evening (%)	14.2	8.0	7.0	8.0	5.2	5.7	3.2	0.8

BMI: body mass index (kg/m^2). Data are presented as mean ± SD, or percentage where specified. All subjects who declared they take sleep medication (prescribed or not) were not considered as "not complaining", so they were not included in the analysis. If analysis showed a significant effect, it was specified as: (1) – gender differences; (2) – age differences; (3) – age x gender differences, $p<0.05$.

Table 2. Effects of age on chronotype.

	50-59 y.o. vs 40-49 y.o.		60-69 y.o. vs 40-49 y.o.		70-79 y.o. vs 40-49 y.o.	
	95% CI	**p value**	**95% CI**	**p value**	**95% CI**	**p value**
Chronotype		0.002		p<0.0001		p<0.0001
Morning type	1.89[1.38; 2.59]		3.04[2.16; 4.27]		7.62[4.36; 13.31]	
Neither	1.36[1.04; 1.80]		1.46[1.08; 2.00]		2.89[1.67; 5.00]	
Evening type	1		1		1	

Multinomial logistic regression, unadjusted model; "evening type" was used as reference for chronotype; "definite morning" and "moderate morning" were unified in "morning type" category; also, "evening type" resulted from unification of "definite evening" and "moderate evening" participants.

Table 3. Effects of age and gender on variables assessed by polysomnography

	40-49.9y.o.		50-59.9y.o.		60-69.9y.o.		70-80y.o.	
	women	**men**	**women**	**men**	**women**	**men**	**women**	**men**
Total sleep time **(2)**	434.63 ± 57.2	414.5 ± 55.4	433.18 ± 51.2	400.99 ± 56.3	430.60 ± 59.8	423.60 ± 59.9	425.60 ± 49.9	410.83 ± 61.6
Sleep efficiency (%) **(1,2,3)**	91.50 ± 4.6	91.27 ± 4.2	91.06 ± 4.2	89.63 ± 4.4	88.98 ± 4.5	88.02 ± 4.7	86.28 ± 4.8	87.86 ± 4.2
Wake after sleep onset **(1,2,3)**	41.11± 24.3	40.33± 21.7	43.13 ± 22.1	47.23 ± 22.7	54.26 ± 25.4	58.07 ± 25.1	67.9 ± 24.8	56.73 ± 24.3
Sleep onset latency (to any stage) **(2)**	15.23 ± 19.8	12.90 ± 15.6	15.50 ± 19.1	13.98 ± 14.5	16.5 ± 20.1	12.30 ± 14.1	19.69 ± 16.8	9.01 ± 24.3
Sleep onset latency (to stage 2) **(2)**	18.95 ± 18.7	17.37 ± 16.9	18.71 ± 19.6	17.86 ± 15.2	19.5 ± 21.3	15.40 ± 14.1	22.75 ± 16.8	11.75 ± 10.2
Latency to SWS **(1)**	21.59 ± 22.3	21.05 ± 17.2	17.01 ± 12.9	22.22 ± 4.8	26.93 ± 26.4	34.95 ± 60.9	27.64 ± 25.8	28.7 ± 31.6
Latency to REM sleep	82.21 ± 35.6	80.04 ± 31.5	83.50 ± 40.7	77.92± 30.8	77.06 ± 36.7	76.28 ± 36.6	88.75 ± 53.1	70.73 ± 22.6
Stage 1 % **(1,2)**	8.37 ± 3.8	10.30 ± 4.3	8.81 ± 3.9	11.97 ± 4.7	8.98 ± 3.4	11.51 ± 6.0	9.24 ± 4.6	13.11 ± 4.7
Stage 2 % **(1)**	43.66 ± 7.4	44.72 ± 7.5	44.09 ± 7.5	44.29 ± 7.3	47.52 ± 8.6	48.76 ± 10.2	48.14 ± 11.7	50.45 ± 12.9
Slow wave sleep % **(1,2)**	22.64 ± 7.7	21.92 ± 6.9	23.34 ± 7.7	21.60 ± 7.8	20.41 ± 6.8	17.28 ± 7.8	20.53 ± 6.9	16.93 ± 7.8
REM sleep (%) **(1,2)**	25.36 ± 4.5	23.05 ± 4.9	23.73 ± 4.8	22.12 ± 4.8	23.07 ± 5.3	22.43 ± 5.1	22.08 ± 4.5	19.48 ± 5.0
Oxygen desaturation index 4% **(1,2)**	1.62 ± 2.4	4.11 ± 3.7	3.03 ± 3.02	4.97 ± 4.0	4.07 ± 3.6	5.84 ± 4.1	4.34 ± 3.5	6.43 ± 4.1
Periodic leg movements index during sleep **(1)**	1.76 ± 3.5	2.15 ± 3.8	2.31 ± 3.77	2.33 ± 3.9	3.12 ± 4.6	2.61 ± 3.8	3.01 ± 4.2	5.28 ± 5.5
Total arousal index **(1,2)**	14.53 ± 5.7	17.80 ± 7.0	14.81 ± 5.8	18.26 ± 5.7	16.45 ± 5.8	19.47 ± 3.9	17.24 ± 5.6	21.36 ± 4.9
Spontaneus arousals **(1)**	10.69 ± 4.2	10.60 ± 4.7	9.80 ± 4.3	9.73 ± 4.3	10.32 ± 4.5	10.38 ± 5.2	10.32 ± 3.9	11.72 ± 4.7
Respiratory arousal index **(1,2)**	2.02 ± 2.4	4.88 ± 4.5	3.21 ± 3.3	6.31 ± 4.7	4.05 ± 3.3	7.43 ± 6.7	4.93 ± 3.7	7.74 ± 4.8
Periodic leg movements arousals **(2)**	0.78 ± 1.8	0.61 ± 1.5	0.71 ± 1.44	0.44 ± 0.9	1.07 ± 1.9	0.62 ± 1.2	1.23 ± 1.8	1.38 ± 1.9

Two-way ANOVA with factors "age group", "gender" and interaction (N=860) for the main polysomnographic variables. Significant effect was specified as: (1) – gender differences; (2) – age differences; (3) – age x gender differences, $p<0.05$.

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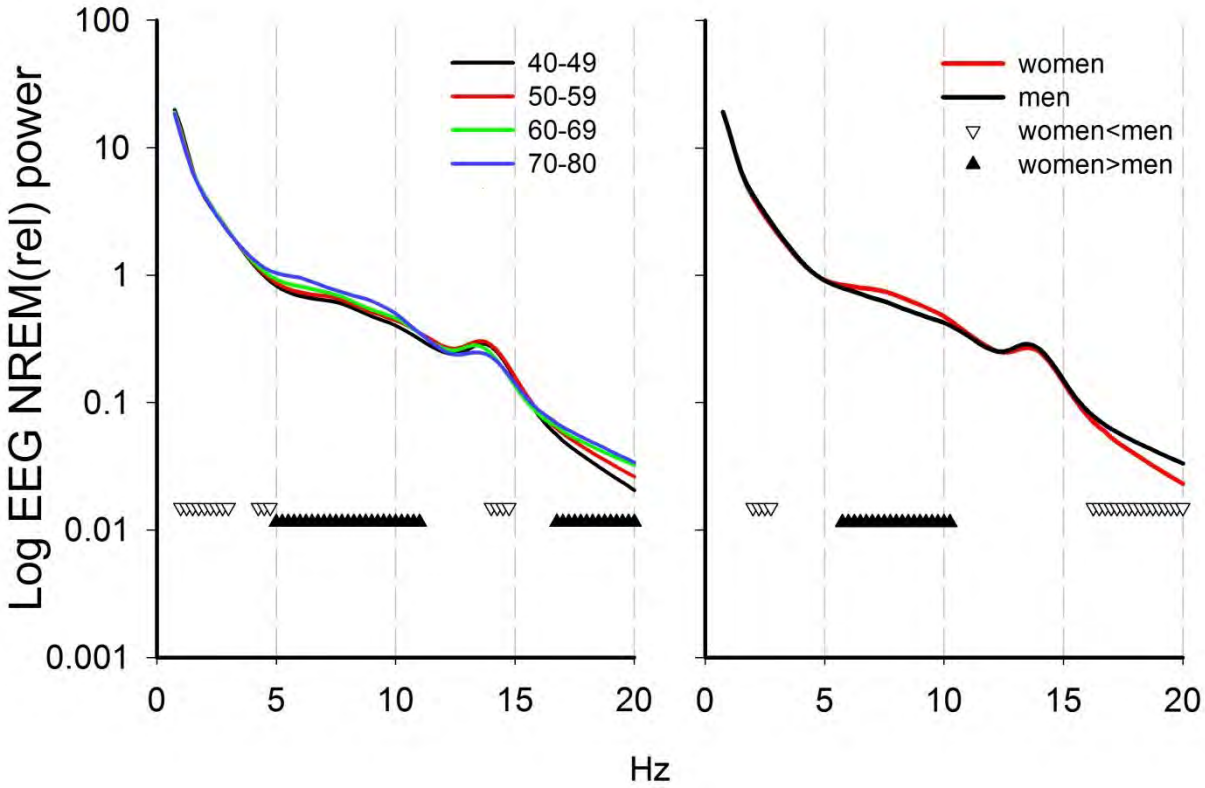

Figure 1. Effect of age on NREM relative EEG spectral power (left). Down oriented triangles connect significant frequencies which were decreased with age, while up-oriented triangles connect significant frequencies which were increased with age ($p<0.05$). Effect of gender (right) on NREM relative EEG spectral power.

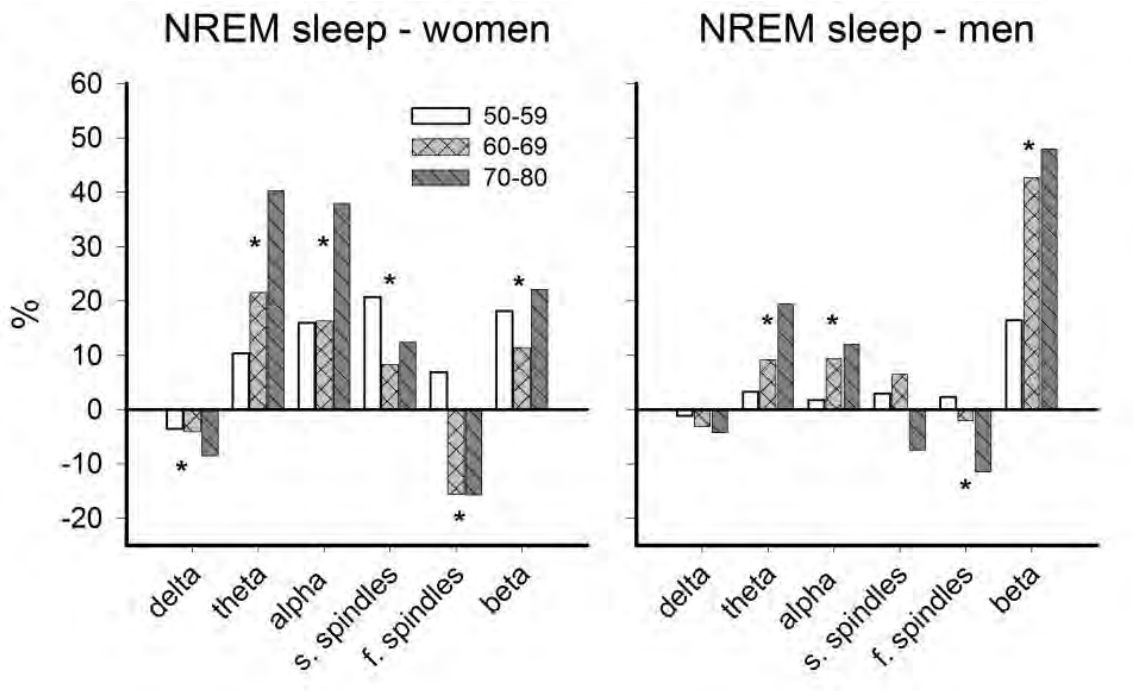

Figure 2. Effect of age on NREM EEG frequency bands (relative to the first age group) separately for men and women. Stars mark EEG bands significantly affected by age ($p<0.05$); s. spindles= slow spindles; f. spindles= fast spindles.

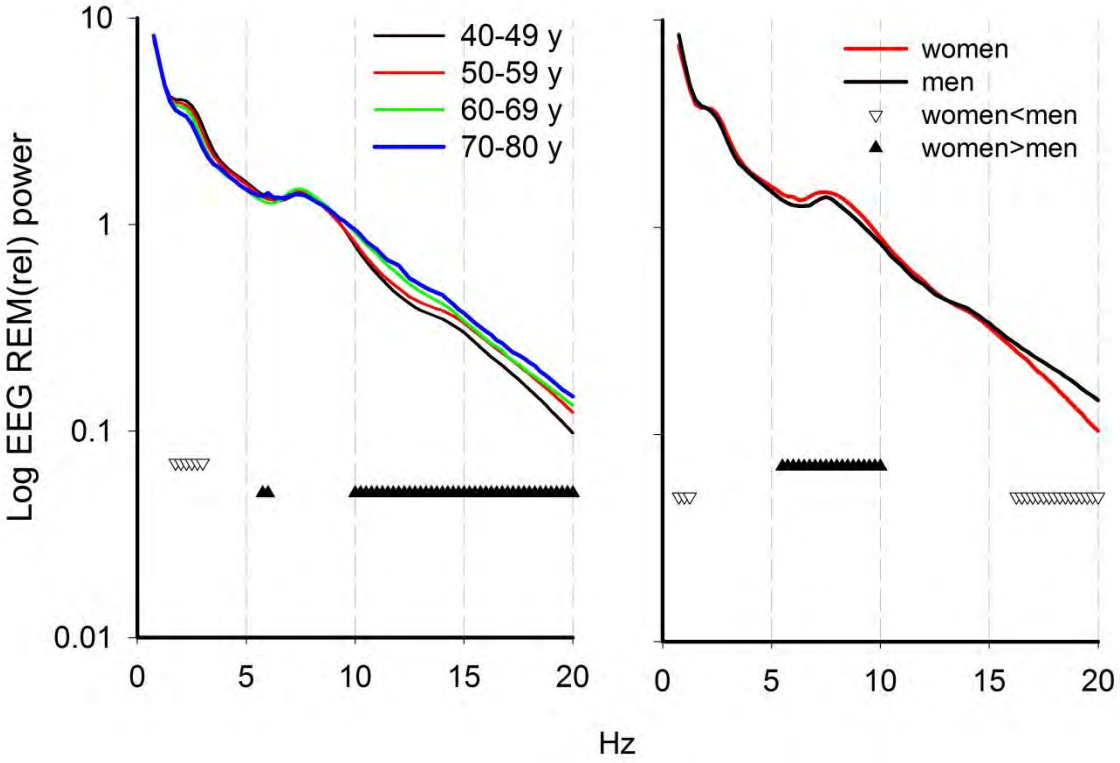

Figure 3. Effect of age (left) and gender (right) on REM sleep spectral composition. Down oriented triangles connect significant frequencies which were significantly decreased with age, while up-oriented triangles connect significant frequencies which were significantly increased.

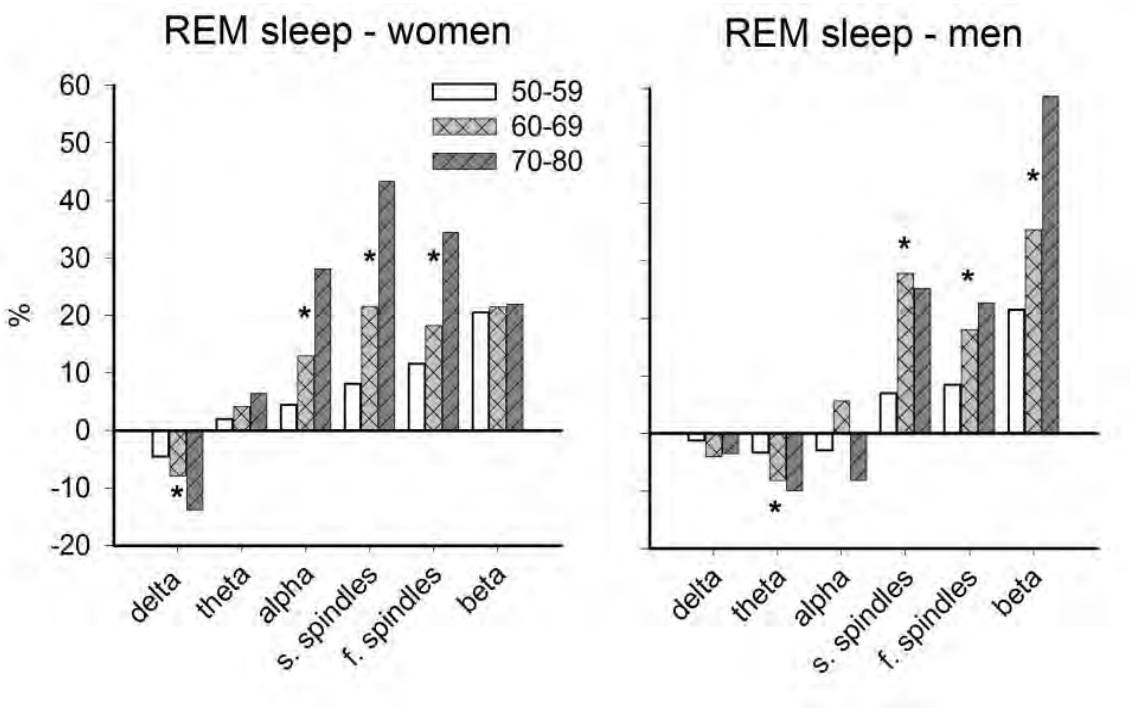

Figure 4. Effect of age on REM sleep EEG frequency bands (relative to the first age group) separately for men and women. Stars mark bands significantly affected by age ($p<0.05$); s. spindles= slow spindles; f. spindles= fast spindles.

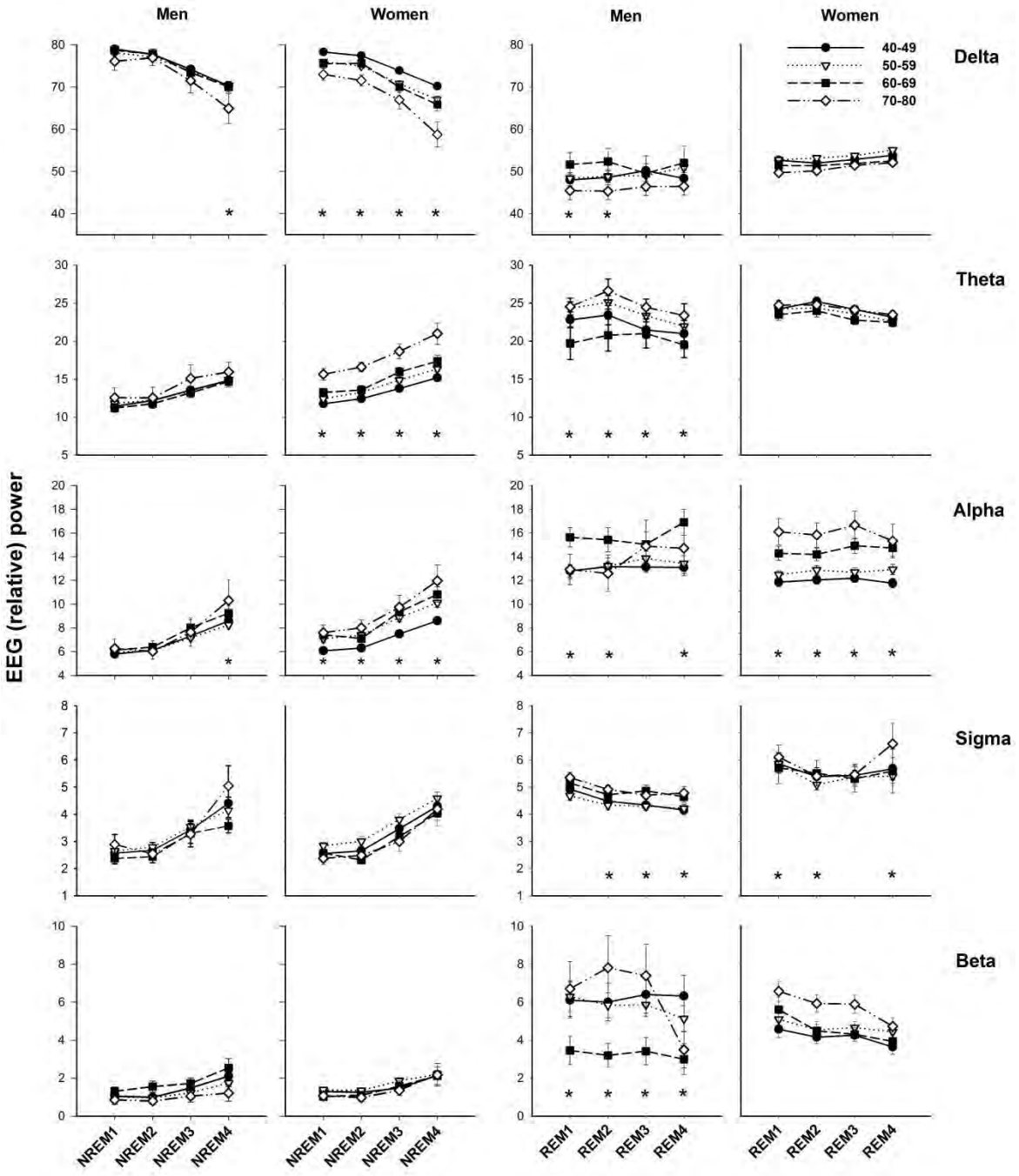

Figure 5. Effect of age on NREM (left) and REM spectral power densities across sleep cycles. Stars connect age groups which differ within the sleep cycles (two-way ANOVA for “cycle”, “age group” and interaction, $p<0.05$).

Central and peripheral metabolic changes induced by gamma-hydroxybutyrate

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Abstract

Study Objectives: Gamma-hydroxybutyrate (GHB) was originally introduced as an anesthetic but was first abused by bodybuilders and then became a recreational or club drug.[1] Sodium salt of GHB is currently used for the treatment of cataplexy in narcolepsy patients. The mode of action and metabolism of GHB is not well understood. GHB stimulates growth hormone release in humans and induces weight loss in treated patients, suggesting an unexplored metabolic effect. In different experiments the impact of GHB administration on central (cerebral cortex) and peripheral (liver) biochemical processes involved in the metabolism of the drug, as well as the effects of the drug on metabolism were evaluated in mice.

Design: C57BL/6J, $GABA_B$ knock-out and obese (ob/ob) mice were acutely or chronically treated with GHB at 300mg/kg.

Measurements and Results: Respiratory ratio decreased under GHB treatment, independent of food intake, suggesting a shift in energy substrate from carbohydrates to lipids. GHB treated C57BL/6J and $GABA_B$ null mice but not ob/ob mice gained less weight than matched controls. GHB dramatically increased the corticosterone level but did not affect growth hormone or prolactin. Metabolome profiling showed that an acute high dose of GHB did not increase the brain GABA level. Both in the brain and the liver, GHB was metabolized into succinic semialdehyde by hydroxyacid-oxoacid transhydrogenase. Chronic administration decreased glutamate, s-adenosylhomocysteine, oxidized gluthathione, and increased omega-3 fatty acids.

Conclusions: Our findings indicate large central and peripheral metabolic changes induced by GHB with important relevance to its therapeutic use.

Keywords: GHB, brain, liver, metabolomics, anti-oxidant

Introduction

Gamma-hydroxybutyrate (GHB), a short fatty acid derivative of GABA, is an inhibitory neurotransmitter, naturally present in the mammalian brain[2, 3]. In sleep medicine, sodium oxybate, a sodium salt of GHB, is used for the treatment of narcolepsy with cataplexy [4]. GHB is an agonist of $GABA_B$ receptors but its mode of action and the range of effects are not well understood [5]. Originally introduced as an anesthetic in early sixties [2], GHB was soon proposed to produce a physiological sleep with increased slow wave sleep (SWS) [6]. However, studies in animals suggested that the GHB-induced changes mimicked the state of absence seizure with abnormal electroencephalogram (EEG) [7, 8]. More recently, we extensively studied the effects of GHB on the EEG and vigilance states in mice and humans [5, 9]. GHB dose-dependently increased EEG slow waves but at higher doses the EEG and behavior were very similar to classical anesthetics [5]. We also showed that both in humans and mice, the GHB-induced behavioral changes were different from physiological sleep. Finally, we showed that exogenous GHB acts solely through $GABA_B$ receptors since GHB had no behavioral and/or EEG effects in $GABA_B$ knock-out (KO) mice [5].

An increase in growth hormone, simultaneously with the first slow wave sleep episode was reported in healthy young volunteers [10] and in narcolepsy patients treated with sodium oxybate [11]. The increase in growth hormone is believed to be the reason why GHB was commonly abused by bodybuilders [12]. GHB is also abused as a recreational drug and was associated with euphoria, behavioral disinhibition, dizziness, myoclonus, retrograde amnesia (date rape drug), nausea and vomiting, confusion, and coma [13]. GHB overdose, both in fatal and non-fatal cases, presents with respiratory depression, bradycardia, and hypotension. [14]

Given the major behavioral effects of GHB, mostly central actions of GHB have been studied, although GHB is also present at periphery and might have several actions. For instance, one of the side effects observed in GHB treated narcolepsy [15], fibromyalgia [16], and binge eating disorder [17] patients is weight loss, suggesting metabolic effect that remains ill-defined. Early studies on GHB central mode of action pointed to a dose-dependent reduction of cerebral glucose utilization in the rat brain [6, 18, 19], which was interpreted as an adaptation to decreased brain metabolic needs. GHB was also tested for potential protective properties in animal models of stroke and hypoxia [20, 21], head trauma [22], and transient global cerebral ischemia [23]. Also, GHB administration induced hypothermia in rats by decreasing metabolic heat production. Nevertheless, it is not understood how these metabolic effects are produced. It was proposed that GHB shifts the intermediary metabolism toward the pentose-phosphate pathway (PPP) by activating glucose-6-phosphate dehydrogenase [24]. As most other central depressants, GHB does not increase glucose supply to the brain but decreases its use by widespread neuronal inhibition but whether the same happens at periphery is not known.

Thus, the aim of the present study was to determine which metabolic pathways are affected by acute and chronic administration of GHB and how chronic administration of the drug impacts body weight as well as brain and body composition in different mouse strains.

Methods

Animals: All experiments were performed according to the protocols approved by the State of Vaud Veterinary Office, Switzerland. All mice were male and kept in individual polycarbonate cages under 12 h light/dark cycles, at 25°C. $GABA_B$ KO mice (lacking either $GABA_{B1}$ or $GABA_{B2}$ subunit, generated in BALB/c background) were generously provided by Dr. Bernard

Bettler (University of Basel, Switzerland) and were generated as previously described [5, 25]. Both $GABA_{B1}$ and $GABA_{B2}$ -/- mice do not have functional $GABA_B$ receptors. Ob/ob mice (C57BL/6J background, leptin deficient due to a spontaneous obese "Ob" mutation) were purchased from Charles River laboratory.

Experimental design: To evaluate the metabolic effects of GHB, we designed 3 different experiments to assess, separately, the acute, short term, and chronic metabolic effects of the drug (Fig. 1). We chose 3 different genotypes: C57BL/6J mice as wild type (and the strain commonly used for metabolic studies), $GABA_B$ KO mice due to the fact that previous research showed these mice are behaviorally insensitive, in terms of sleep and locomotor activity, to GHB[5], and ob/ob mice to test if the effects found in the previous two genotypes are leptin-related.

Experiment 1: Acute effects of GHB administration

To test the acute metabolic changes, blood and tissue metabolites were assessed 1 hour after the drug administration. C57BL/6J mice (N=16, age 12 weeks) were split in two groups: treatment and placebo. Drug (300 mg/kg of Xyrem®, oral solution, 500mg/ml, UCB Pharma SA, Basel, Switzerland, hereinafter "GHB") or placebo (NaCl 0.9%, B.Braun Medical AG, Emmenbrücke, Switzerland) were administrated intraperitoneally (i.p.) with a volume of 5ml/kg body weight. The dose and the duration were based on previous experiments performed in our lab[5], which showed that the maximum effects were obtained at 300 mg/kg and lasted for approximately 1 hour. Blood and tissues were collected one hour after GHB/placebo administration. Blood cholesterol, triglyceride, glucose, and free fatty acids were measured. The brain and liver were removed, stored at -80°C and used for metabolome analysis.

To evaluate the effects of the drug on corticosterone, prolactin, and growth hormone we

used the same protocol but in two genotypes: C57BL/6J and $GABA_B$ KO mice (lacking either $GABA_{B1}$ or $GABA_{B2}$), age 12-13 weeks. One hour after i.p. injection of 300mg/kg of GHB, animals were rapidly decapitated and trunk blood was collected, centrifuged at 1,000g for 15 min at 4°C and the supernatant stored at -80°C. Corticosterone was quantified by an EIA kit (Enzo Life Sciences AG, Lausen, Switzerland) according to the manufacturer instructions. Test samples (duplicates) were diluted 40 times in the provided buffer, and optical density was measured (λ = 405 nm). Prolactin and growth hormone were measured by ELISA kits (Mouse prolactin ELISA kit, Sigma Aldrich, St. Louis, MO, USA and Rat/Mouse growth hormone ELISA kit, EMD Millipore, St. Charles, MO, USA). Test samples (duplicates) were diluted 1:10 in provided buffer and absorbance (λ =450 nm for prolactin and the difference between λ =450nm and 590 nm for growth hormone) was recorded.

Experiment 2: Effects of subchronic administration of GHB

In a second step, mice were studied in the calorimetric chambers for 8 days: 4 baseline days and 4 days under drug administration. After one day of habituation, 10 C57BL/6J mice, 12 weeks old, were recorded for 2 sessions of 96 hours in calorimetric chambers (Oxymax). First group of 5 mice received 300 mg GHB/kg/day in drinking water, starting at first evening. The second group of 5 mice received the same dose of drug in the same manner, from hour 97 of the experiment (balanced design). Placebo (water) was administered on the opposite order. At the beginning of the experiment mice were weighed, and measured parameters were adjusted for each mouse weight. Energy expenditure (EE) was calculated as: $3.815*VO_2 + 1.232*VCO_2$ [26]. Locomotor activity, oxygen consumption (VO_2) and CO_2 production (VCO_2), respiratory exchange ratio (RER), food and water intake and heat production were calculated by Oxymax software.

Experiment 3: Effects of chronic GHB administration

For chronic metabolic changes, mice were studied 4 weeks under placebo or 300 mg GHB/kg/day. Twenty C57BL/6J mice and 16 ob/ob mice were split into two groups, balanced for their age (range: 12 weeks old at the beginning of the experiment) and weight. One week before the experiment all mice were habituated to drink water only during light (sleep) period to mimic the administration of the drug in humans; water bottles were removed at 9.00 pm and reinstalled at 9:00 am; and the daily quantity of water was measured. After 1 week of habituation, mice were weighed and split in two groups. Treated group received in the drinking water 300 mg GHB/kg/day, diluted in 4 ml of water. All mice consumed this quantity of water. Control mice received water only during the light period. Lean mass, fat mass and water content were assessed on isoflurane-anesthesized animals using an EchoMRI whole-body composition analyzer. Mice were evaluated for body composition at the beginning of the experiment and after 28 days of treatment. Weight was monitored weekly using an electronic scale. To avoid the effects of isoflurane on metabolites analysis, after the second EchoMRI measurement drug/water administration continued for another 24h. At the end of the experiment, mice were sacrificed by cervical dislocation. Brain and liver of C57BL/6J mice were rapidly sampled, stored at -80°C and used for metabolome analysis.

300 mg GHB/kg/day, diluted in 4 ml of water was also administrated for 4 weeks, during the light period, to 4 $GABA_{B1}$ and 5 $GABA_{B2}$ KO mice and placebo to 4 $GABA_{B1}$ and 3 $GABA_{B2}$ KO mice, matched for age (12-13 weeks) and weight at the beginning of the experiment. Before starting the experiment mice were habituated for 1 week to have access to water only during the light period. Weight was monitored weekly.

For metabolome analysis, the cortex was dissected from the frozen brains. A sample of

100-140 mg from frozen liver was also collected. All samples were stored at -80°C till processing. Sample preparation and analysis were performed by Metabolon, as previously described [27].

Statistical analyses: To test the effects of the treatment on blood biochemistry, calorimetric variables and body mass composition, paired t-test or Wilcoxon signed rank test were used, when appropriate. Weekly changes in weight were analyzed by One-way repeated measures ANOVA. To identify the impact of light/dark variability, effects of treatment, order or their interaction, two-way ANOVA was used. Evaluation of the relationship between body mass composition and weight changes was analyzed by Deming regression (errors-in-variable model), and then the slopes were compared by t-test. Calorimetric data were normalized to the first 4 hours of the experiment. Metabolomics data were analyzed by Welch t-test and fold change (log2(treated/control)) and significance level (-log10(p value)) were calculated.

Results

Experiment 1

Corticosterone is increased by GHB

One hour after drug administration, no statistical differences were found in plasma concentrations of glucose, cholesterol, triglyceride, and free fatty acids. Corticosterone levels were significantly increased in both C57BL/6J and $GABA_B$ KO treated mice (almost 4-fold), independent of genotype (two-way ANOVA, $p<0.001$ for factor "treatment", non-significant for genotype and interaction, C57BL/6J=17, $GABA_B$ KO=7) (Fig. 2*a*). Prolactin was not affected by GHB administration, in none of the genotypes (C57BL/6J=18, $GABA_B$ KO=6). Growth hormone

was slightly (though not significantly) decreased by GHB in both genotypes (C57BL/6J=20, $GABA_B$ KO=7). Nevertheless, $GABA_B$ KO mice had significantly higher values compared to C57BL/6J, independent of the treatment (Fig. 2*b*).

Acute metabolic effects of GHB

Metabolomics was used to evaluate the impact of GHB administration on central (cortex) and peripheral (liver) biochemical processes involved in the metabolism of the drug, as well as the effects of the drug on metabolism. Overall, 226 metabolites could be identified in the cortex and 314 in the liver. Supplemental Table S1 shows all detected metabolites with those that display significant differences between treated and control groups highlighted.

One hour after administration, GHB was found at over 300-fold higher levels in the brain and nearly 200-fold in the liver. The major degradation pathway activated was GHB oxidation to succinic semialdehyde catalyzed by hydroxyacid-oxoacid transhydrogenase[28]. The rate of catabolism was higher in the liver than in the brain (2-hydroxyglutarate raised 29-fold in the liver but only 3-fold in the brain), but surprisingly glycolates as the final breakdown products were at 7-fold in the brain and 5-fold in the liver. Interestingly, GABA was not changed despite the large increase in GHB, strongly suggesting that GHB is not converted to GABA even at such high doses.

Glucose and many of the glycolysis metabolites glucose-6-phospahate, fructose-6-phosphate, fructose 1,6-diphosphate/glucose 1,6-diphosphate, were significantly increased in the cortex of GHB-treated mice. In GHB-treated mice there was a significant increase in pyruvate and acetyl-CoA, but metabolites of the TCA cycle, citrate and malate, were

significantly decreased and, although not statistically significant, fumarate levels were also reduced. The limiting factor for acetyl-CoA entrance into the TCA cycle is oxaloacetate, which was not measured in these samples.

A surprising finding was a large increase in lysolipids in the cortex of GHB-treated mice. Whether this indicates an increased membrane remodeling or an inhibition of lipid reacylation is difficult to disentangle. Long-chain acylcarnitines were also significantly increased but the ketone body 3-hydroxybutyrate (BHBA) that is made during β-oxidation was actually decreased.

As opposed to the cortex, most detected metabolites in the liver were decreased in acutely GHB-treated mice. Amongst few exceptions, corticosterone was found at 4-fold higher levels after GHB. A major pathway affected by GHB in the liver was the bile acid metabolism, with almost all detected metabolites significantly decreased in GHB-treated mice. Although this finding may suggest a reduced lipid processing, no major changes in lipids were detected, except for an increase in docosapentaenoate (22:5n6). There was also a general decrease in amino acids (except for the BCAAs, as indicated above), peptides, and carbohydrates metabolism.

Experiment 2

Respiratory ratio is reduced by GHB

For the light period during the calorimetry, the group of C57BL/6J mice who received the treatment first, did not show any significant differences between the treatment and control sessions. For the second group (which received the placebo first and then the drug), a significant decrease in heat production (paired t-test, t= -2.51, p=0.01), CO_2 elimination (VCO_2) (paired t-

test, t= -2.78, p=0.006), O_2 consumption (VO_2) (paired t-test, t= -2.92, p=0.004) and energy expenditure (EE) (paired t-test, t= -2.91, p=0.003) was observed. Due to proportional decrease in CO_2 and O_2, respiratory exchange ratio (RER) was not significantly different.

During the dark period, a significant decrease for Z axis activity (exploratory and possibly drinking – paired t-test, t= -2.75, p=0.007) and RER was observed for the first group, while in the second group, there was a significant decrease in feeding (Wilcoxon signed rank, W=2153, p<0.001) and RER (paired t-test, t= -2.63, p=0.009). There was no significant reduction in locomotor activity between the two conditions. In spite of between group differences for locomotor activity on Z axis, RER and feeding, analysis of the effect of order, treatment and their interaction for the dark period, indicated that the only variable which changed was RER (two-way repeated measures ANOVA with factors "treatment" p<0.001, "order", p=0.36, and "treatment × order", p=0.793).

Calculated over 24h, paired t-test (Wilcoxon signed rank) for the first group showed no differences between treatment and control conditions for activity, feeding, heat production, VO_2 or VCO_2. The only parameter, which decreased significantly was again RER (Wilcoxon signed rank test, W=4833, p=0.003), and this decrease was independent of food intake. For the second group, feeding, VO_2 (paired t-test, t=-2.569, p=0.01), VCO_2 (t=-3.594, p<0.001) and RER (paired t-test, t=-1.997, p=0.04) were decreased. Again, when taking into account both, "treatment" and "order" effects, the decrease in RER was influenced by the treatment only (two-way repeated measures ANOVA with factors "treatment" p=0.04, "order" p=0.4 and "order by treatment" p=0.79). The RER changes over 96h of the experiment are reported in Figure 3. No changes in weight were observed. All calorimetric results are shown in Supplemental Table S2.

Experiment 3

Body composition is changed by GHB

There were no significant differences between treated and control groups for fat mass, lean mass, or water content evaluated by EchoMRI after 4 weeks of treatment. Statistical tests were performed on both absolute and relative values (calculated as percentages as compared to baseline). Nevertheless, the regression analysis followed by t-test showed a more balanced distribution of fat mass and lean mass in treated compared to control mice ($t= 9.30$, $p<0.001$). The higher the lean mass increase, the lower was the fat content. This suggests that in treated group weight loss is determined by initial fat content; body fat content is a determinant of the relative proportion of lean tissue loss, but the weight change is inversely correlated with relative increase in lean mass (tests for slopes equal 0 and 1, respectively, $p<0.005$ in treated mice and non-significant in control mice). Similar experiment performed in obese (ob/ob) mice showed a comparable trend, although not significant (due to a smaller change relative to their weight).

Slower weight gain with GHB

During chronic (4 weeks) administration of GHB, C57BL/6J treated mice gained less weight than matched controls (Figure 4). Note that at this age, weight curve is still increasing in both groups (two-way repeated measures ANOVA with factors "treatment" $p=0.001$, "week", $p=0.002$, and "treatment × week", $p=0.27$) (Fig. 4*a*). The same treatment in ob/ob mice showed a similar trend but non-significant (t-test, $P=0.27$, Fig. 4*b*). To test if the decrease in weight observed in treated C57BL/6J mice was related to a reduction in activity level or food intake, we administrated the drug in $GABA_B$ KO mice, who are behaviorally insensitive to GHB in terms of behavior and sleep-wake parameters[5]. The analysis was performed after 3 weeks of treatment in 11 mice (6 treated and 5 controls, only 3 weeks are considered since 5 mice died during the

fourth week due to seizures). A significant decrease in weight was observed in treated mice (two-way repeated measures ANOVA with factors "treatment" p=0.002, "week", p=0.20, and "treatment × week", p<0.001) (Fig. 4*c*). The weight loss observed in $GABA_B$ KO mice suggests that the effect is not centrally mediated through $GABA_B$ receptors.

Chronic metabolic effects of GHB

Overall, chronic GHB administration showed a different metabolic effect. As opposed to the acute administration, GHB levels were only marginally increased (Supplemental Table S3) both in the cortex and the liver while 2-hydroxyglutarate (intermediate metabolite) was still increased by 6-fold in the liver. Also, no changes were observed either in the glucose or the BCAA metabolism or their metabolites. This finding was anticipated because of the very short half-life of GHB (10-20min) and the fact that in the acute situation animals were injected with a bolus of 300mg/kg, while they consumed the same dose in their drinking water over the 12h light period during the 4 weeks of experiment. Although most lysolipids were unaffected by the chronic GHB, 1- and 2-linoleoglycerophosphocholines were still significantly increased in the cortex (as in the acute experiment) while significantly decreased in the liver. As in the acute experiment, long-chain acylcarnitines tended to be increased in the cortex, while the ketone body 3-hydroxybutyrate was decreased both in the cortex and the liver (Fig. 5).

S-adenosylhomocysteine was significantly decreased in the cortex (fold change=0.87, p<0.006) and tended to be also decreased in the liver (fold change=0.91, p<0.11). This might have important consequences in terms of methionine and cysteine availability. Interestingly, oxidized glutathione was significantly reduced together with riboflavin, while reduced glutathione tended to increase in the cortex of GHB-treated mice. This finding indicates that

GHB might have some anti-oxidant effects, as already suggested.

GHB treatment increased the nucleotides turnover in the cortex with a significant increase in purinic/pyrimidinic nucleotides formation (inosine 5'-monophosphate, guanosine 5'-monophosphate and cytidine 5'-monophosphate) together with an increase in N-acetylmethionine. Low levels of allantoin, xanthine and xanthosine are in favor of the hypothesis that the increase in nucleotides is not resulting from intense catabolism, but rather from increased turnover.

As opposed to the acute condition, the primary bile acids, conjugated or not, were unchanged in the liver after 4 weeks of GHB treatment. However, the levels of conjugated secondary bile acids (taurodeoxycholate and 6-β-OH-lithocholate) tended to increase (fold changes from 1.22 to 1.50, p values from 0.2 to 0.08), suggesting a role of transformation of biliary acids in the intestines and their reuptake for transport to the bile. Chronic GHB treatment led to the elongation of fatty acids in the liver (higher stearidonate, important for polyunsaturated fatty acids formation, as well as docosadienoate, and 12,13-hydroxyoctadec-9(Z)-enoate), and lower phosphopantetheine, suggestive of an increased fatty acids synthesis. Interestingly, treated mice had an increased level of omega-3 fatty acids, with known beneficial effects. An increase in oligosaccharides metabolism (high maltotriose), especially galactose, and a decrease in fructose were also observed. Tagatose, involved in the regulation of glycemic status and contributing to weight loss, had also higher levels in treated mice. An alternative energy pathway might be activated, with the use of glycerate.

Discussion

Evaluation of multiple variables by indirect calorimetric measurements indicated a significant decrease in RER, a good marker for substrate utilization for energy production. RER is calculated as the ratio between VCO_2 and VO_2, and is used to estimate the relative proportion of different substrates used to produce energy. Expected values of this parameter range between 0.7 and 1, with 1 representing pure carbohydrate use and 0.7 pure lipids use. RER values higher than 1 are associated with lipid synthesis and values lower than 0.7 are indicative of carbohydrates synthesis or ketone body metabolism[29]. Small changes in RER cover significant changes in substrate used: at 0.95, there are 82.9% carbohydrates and 17.1% fat use, while at 0.90, carbohydrates represent 65.9% and fat 34.1%.[30]

The decrease in RER was most significant during the dark period when the mice are active. The differences between the treatment and placebo were bigger in the second group (placebo given before GHB). The RER decreased progressively over the 4 days of drug administration and recovered also progressively during the 4 days of placebo administration, leading to a more pronounced effect when the drug was administered after the placebo. The presence of a metabolic effect after drug administration seems to be independent of locomotor activity and feeding. Decreased heat production observed in the second group may also be a consequence of increase in sleep time. Overall, these results suggest that fatty acids are preferred to glucose as energy substrate under GHB. An increase in lipolysis and a reduction in endogenous glucose production were also described in GHB treated narcolepsy patients after 3 months.[31]

As expected for the age range of the mice, both GHB treated and control animals followed a normal ascending growth curve, but treated animals gained significantly less weight.

Body composition evaluation was a continuation of the calorimetric experiment, since differences in energy expenditure can be explained by difference in lean mass [32]. Treated mice showed a more balanced distribution of body composition, and the differences in weight gain were not due to reduced fat mass, but to increased lean mass. The relationship between lean mass and weight change may point to the involvement of growth hormone in the changes in body composition. GHB in normal subjects and patients with Parkinson disease increased growth hormone [10, 33, 34], and this effect was mediated by cholinergic mechanisms [34]. However, growth hormone was not modified in our experiments as also not reported by others in rats and dogs [35]. This discrepancy might be due to the dose (ranging from 10mg to over 1g), to the timing of the measure after drug administration (30min or 1h), to the technique (radioimmunoassay or ELISA), or to unknown between species difference. Interestingly, $GABA_B$ KO mice had constitutively higher growth hormone levels, suggesting that these receptors might somehow control the growth hormone production and/or release. Flumazenil ($GABA_A$/benzodiazepine antagonist) and metergoline (serotonin receptor antagonist) were also shown to reduce or block growth hormone stimulation by GHB in humans and suggested a GABAergic-serotonergic mechanism[36, 37]. The differences in weight progression between treated and control mice showed a similar trend in ob/ob mice, while a significant difference in weight was observed in $GABA_B$ KO mice, strongly suggesting that weight changes induced by GHB might not be centrally mediated through either growth hormone, leptin, or $GABA_B$ receptors. Also, we showed here that even high doses of GHB do not increase GABA that could act through $GABA_A$ receptors. The lack of significant changes in leptin deficient mice may be due to a ceiling effect (given the morbid obesity in these mice, the GHB doses used here might have reached the maximum effect) or to the fact that these mice might need longer GHB administration to show significant changes. Recent human studies also suggest that metabolic effects of GHB are not leptin or ghrelin mediated[38] and that GHB

treatment does not alter the levels of these two hormones either in narcolepsy patients or in healthy controls.

The large increase in lysolipids and acylcarnitines may suggest membrane remodeling and increased β-oxidation. Nevertheless, unlike in most tissues where acylcarnitines function in β-oxidation, in the brain they are believed to act as a sink for reacylation of phospholipids and triglycerides. The increase in acylcarnitines could be a result of released fatty acids from the phospholipids, and explain why there was no increase in overall fatty acids in the brains of GHB-treated mice. They also may suggest that the proper balance between acyl-CoA, acyl-CoA synthetase, and reacylating enzymes are somehow altered in GHB-treated mice. Further supporting an inhibition of reacylation was the significant increase in mono- and diacylglycerides.

Metabolomics analysis also revealed interesting pathways both in GHB catabolism and affected acutely or chronically by GHB administration. The catabolism of GHB is not well understood. GHB can be metabolized through β-oxidation or converted to succinic semialdehyde either by GHB dehydrogenase or by the more recently identified hydroxyacid-oxoacid transhydrogenase (HOT) [28]. HOT transformation of GHB into succinic semiahdehyde is coupled to the conversion of α-ketoglutarate to 2-hydroxyglutarate and final conversion of succinic semiahdehyde to 4,5-dihydroxyhexanoate, while succinic semialdehyde produced by GHB dehydrogenase is transformed into succinate that enters the TCA cycle. Note that both pathways are connected to the TCA cycle through α-ketoglutarate and succinate. Although we found

changes in all three pathways, the most consistent one was the large increase in 2-hydroxyglutarate, strongly suggesting that the major catabolic pathway is mediated by HOT.

The most impressive change after a single dose of GHB was a dramatic increase in peripheral corticosterone. Most metabolic changes found after acute administration of GHB may be mediated by the elevated corticosterone level. Although we did not measure the activity of the hypothalamic-pituitary-adrenal axis (especially the adrenocorticotropic hormone levels), several results suggest that the effect might not be centrally mediated. Corticosterone levels were increased both in C57BL/6J and $GABA_B$ KO mice after acute i.p. GHB administration. Also, other pituitary hormones, growth hormone and prolactin, were not affected by GHB. As opposed to growth hormone and prolactin that are stimulated in humans but not in other species, cortisol and corticosterone are reliably stimulated by GHB in all species[39, 40]. Whether changes in cortisol induced by GHB may also contribute to the occurrence of depression in susceptible subjects [41] deserves further investigations.

Overall, GHB induced different metabolic changes after acute and chronic administration. Nevertheless, as originally proposed (Laborit, 1964; Wolfson *et al.*, 1977) glucose utilization did not seem to be favored either after acute or chronic GHB administration. The hypothesis that GHB is shifting the energy metabolism to pentose phosphate pathway [24] is only partially supported by our findings. The glucose availability was confirmed by the presence of very low levels of 1,5-anhydroglucitol, a good indicator of glucose levels, which was proposed as a marker for glycemic control in diabetic patients [42]. GHB by inducing a global central inhibition reduces glucose utilization (but not availability), similar to other anesthetics. Normal or increased lactate

levels and decreased ketone bodies in both acute and chronic experiments suggest that the cortex has no deficit in ATP.

The acute changes in BCAA and lysolipids are difficult to explain, although these changes may reflect BCAA utilization as energy substrate and transient activation of phospholipase A2 or inhibition of lipid reacylation. One interesting finding was a general decrease in bile acid metabolism after acute GHB administration. This might suggest a transient reduction in lipid processing resulting in lipid waste. Detailed metabolomics analysis of the serum might be necessary to verify if lipid reabsorption and processing is acutely altered by GHB. One possibility is that acute administration of high dose (with possible anesthetic-like effects on biliary duct and on the receptors from the intestine) blocks the feedback of biliary acids for short term.

Given the short life of GHB, chronic administration resulted in a few different metabolic changes as compared to the acute situation. Decreased glutamate, s-adenosylhomocysteine, oxidized gluthathione, and increased omega-3 fatty acids suggest reduced oxidative stress and improved fatty acid metabolism. GHB was originally developed as an antioxidant agent and early works suggested that GHB protects against severe hypoxia, radiation, and experimental diabetes[43-45]. Although these observations could be replicated, not only for the brain protection but also for peripheral tissues (see review in [46]), contradictory results were also reported, the major source being the large range of GHB doses used in various studies. Generally, when GHB was used at or below 300mg/kg, protective effects were observed while higher doses (500mg/kg or higher) suggested oxidative stress[47]. Nevertheless, a recent study where a single high dose

(1g/kg) of GHB was administered to rats, reported increased expression of genes with neuroplasticity and metabolic effects similar to our findings [48].

In conclusion, GHB has a large and complex metabolic effect with different central versus periphery and acute versus chronic profiles. Overall, changes reported here not only exclude any major toxic effect but instead suggest that GHB might have anti-oxidant, anti-aging, and potentially anti-obesity properties.

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Figure Legends

Figure 1. Study design presenting experiments to determine acute, short-term, and chronic metabolic effects of GHB. *-start of the experiment; ‡ - end of the experiment; [1]- GHB was administrated in the drinking water.

Figure 2. Effects of acute intraperitoneal administration of 300 mg GHB/kg on corticosterone (a) and growth hormone (b) in C57BL/6J and $GABA_B$ KO mice. Corticosterone was significantly elevated in treated C57BL/6J and $GABA_B$ KO mice (two-way ANOVA followed by Holm-Sidak *post hoc* test for "treatment", $p<0.001$; "genotype", $p>0.05$; and their interaction, $p>0.05$, N=20 for C57BL/6J and N=7 for $GABA_B$ KO mice). Growth hormone was not affected by treatment ($p=0.36$), but $GABA_B$ KO mice had significantly higher levels compared with C57BL/6J mice ($p<0.001$ for "genotype", $p>0.05$ for "treatment" and their interaction; N=17 for C57BL/6J and N=7 for $GABA_B$ KO mice). *$p<0.05$ between the treated and the control group; #$p<0.05$ between genotypes.

. **Figure 3**. Effects of GHB administration on RER. Data are presented as percentage of the mean of the first 4h of the baseline. Two-way repeated measures ANOVA for factors "treatment", "time-point", and their interaction, followed by Holm-Sidak *post hoc* test, $p<0.001$ for "treatment" and "time-point"; black lines connect significant different time-points between treated and control mice, $p<0.05$.

Figure 4. Relative weight change calculated as a percentage of the initial weight, in GHB-treated and control groups (black and grey bars, respectively). (a) Two-way repeated measures ANOVA for factors "treatment", "week" and their interaction, followed by Holm-Sidak *post hoc* test showed that the weight gain by C57BL/6J mice in the treated group was

significantly less compared to the control group; *$p<0.05$, N=20. Lines connect weeks for which significant differences were found. (b) The weight gain in ob/ob treated group was lower compared to the control group, but not significantly different between the treated and the control group (N=16). (c) $GABA_B$ KO treated mice lost weight compared with the control mice; *$p<0.05$, N=11.

Figure 5. Main metabolic changes after chronic administration of GHB. On the left side liver metabolic changes are presented, and brain metabolic changes are represented on the right side of the figure. Box plots ($p<0.05$) for some of significant changes are presented in red for liver and blue for the brain. HOT: hydroxyacid-oxoacid transhydrogenase; FA: fatty acids; TCA: tricarboxylic acid; beta-Ox: beta-oxidation; GSSG: glutathione oxidized; GSH: glutathione, reduced; 5'-AMP: adenosine 5'-monophosphate; 5'-CMP: cytidine 5'-monophosphate; 5'-GMP: guanosine 5'- monophosphate; BHBA: 3-hydroxybutyrate.

Figure 1

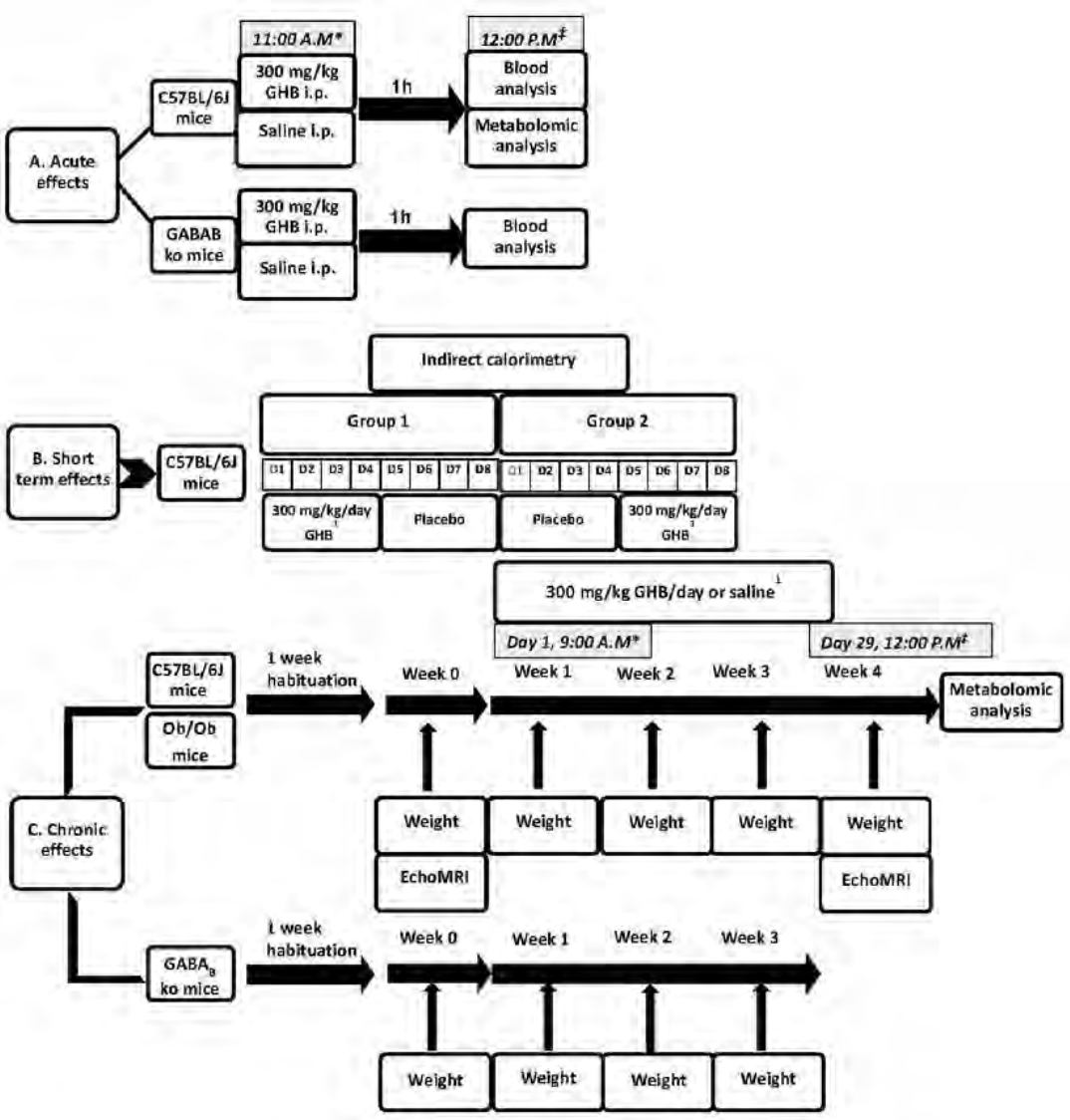

A. Acute effects
C57BL/6J mice
GABAB ko mice
11:00 A.M*
300 mg/kg GHB i.p.
Saline i.p.
1h
12:00 P.M‡
Blood analysis
Metabolomic analysis
B. Short term effects
C57BL/6J mice
Indirect calorimetry
Group 1
Group 2
D1 D2 D3 D4 D5 D6 D7 D8
300 mg/kg/day GHB
Placebo
C. Chronic effects
C57BL/6J mice
Ob/Ob mice
GABA$_B$ ko mice
300 mg/kg GHB/day or saline
Day 1, 9:00 A.M*
Day 29, 12:00 P.M‡
1 week habituation
Week 0
Week 1
Week 2
Week 3
Week 4
Metabolomic analysis
Weight
EchoMRI

Figure 2

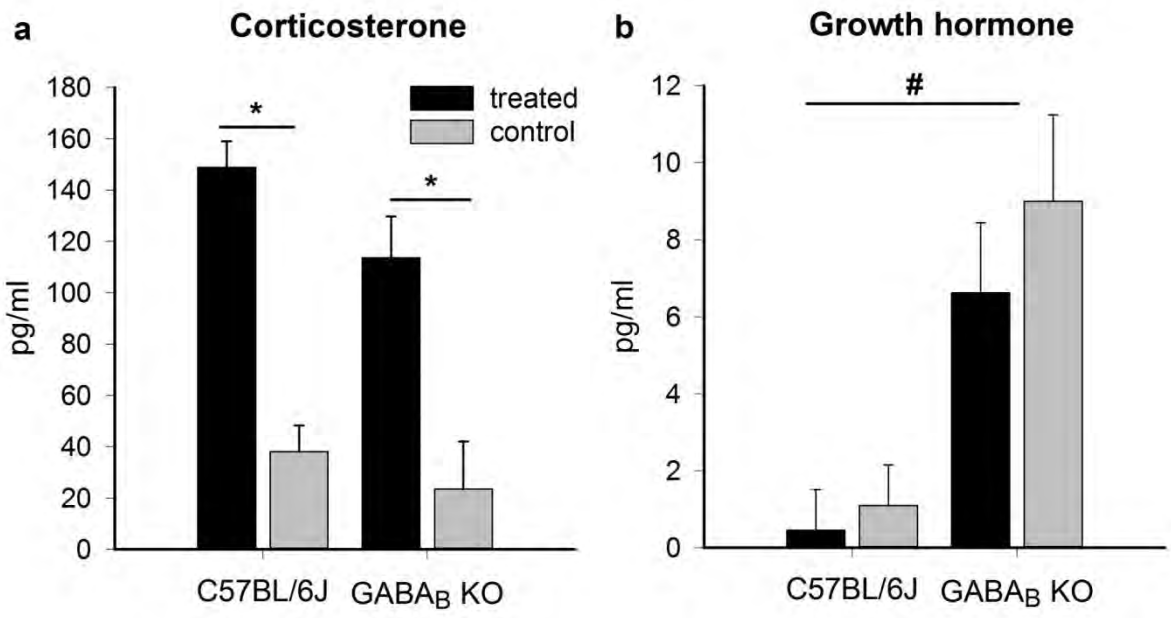

a
Corticosterone
b
Growth hormone
treated
control
pg/ml
180
160
140
120
100
80
60
40
20
0
12
10
8
6
4
2
0
*
*
#
C57BL/6J
GABA$_B$ KO
C57BL/6J
GABA$_B$ KO

Figure 3

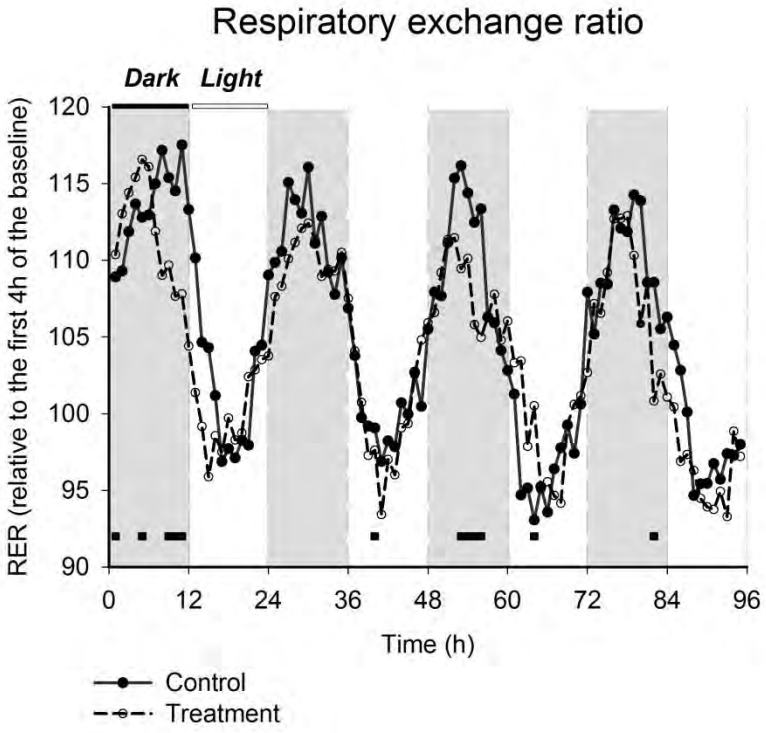

Respiratory exchange ratio
Dark
Light
RER (relative to the first 4h of the baseline)
120
115
110
105
100
95
90
0
12
24
36
48
60
72
84
96
Time (h)
Control
Treatment

Figure 4

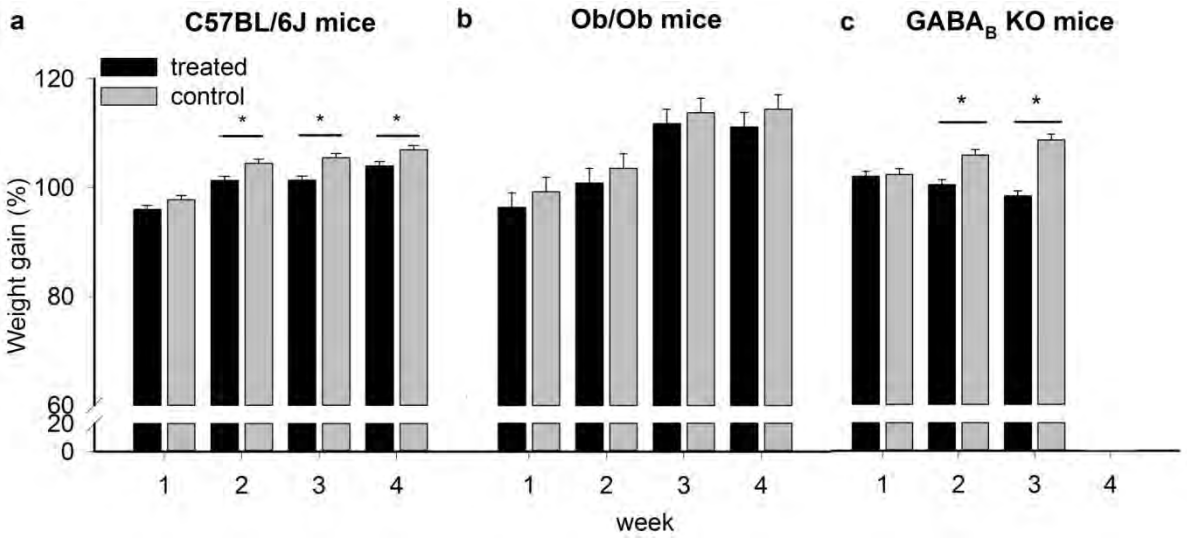
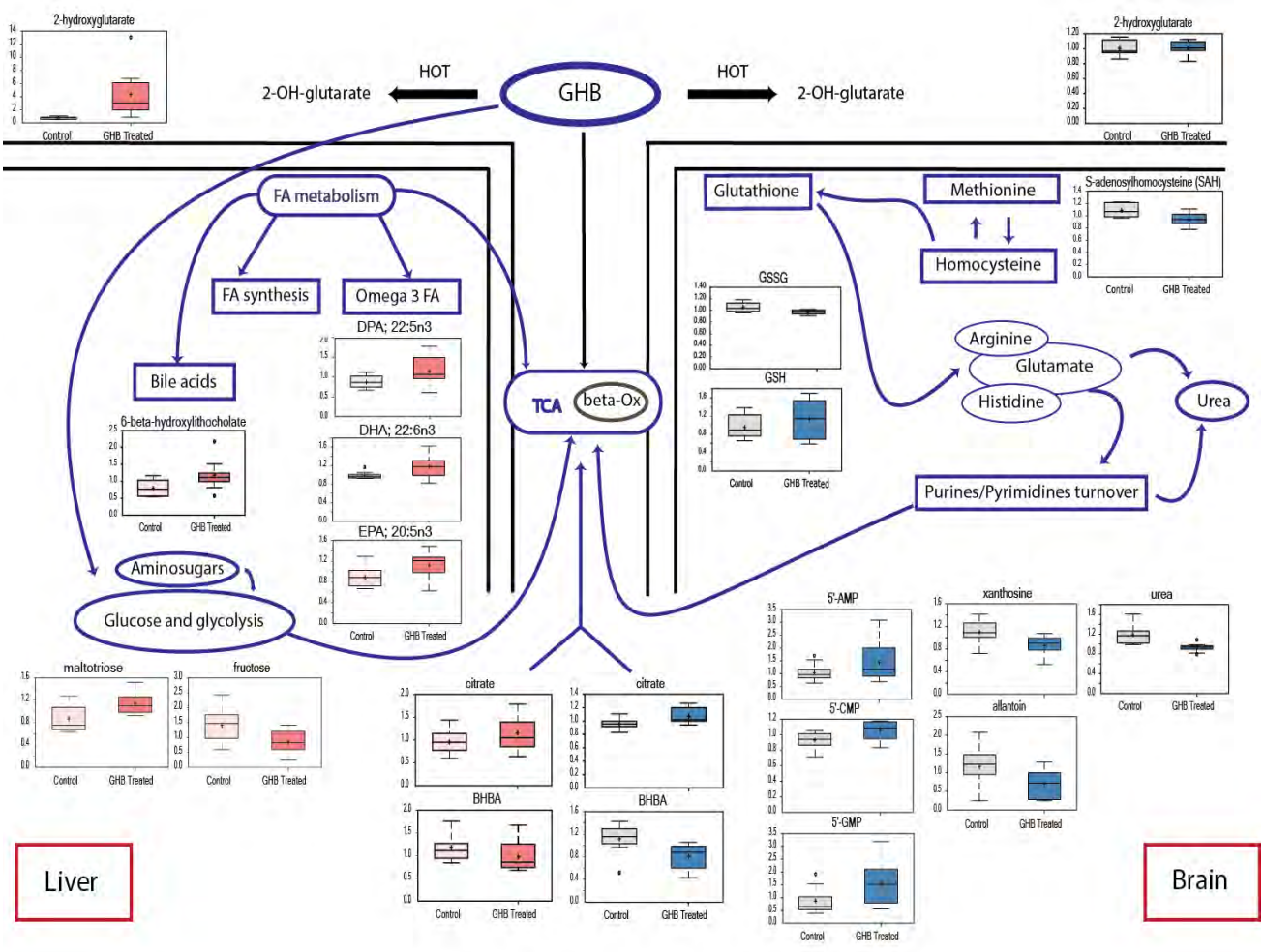

a
C57BL/6J mice
b
Ob/Ob mice
c
GABA$_B$ KO mice
treated
control
Weight gain (%)
120
100
80
60
20
0
*
1
2
3
4
week

Figure 5


2-hydroxyglutarate
Control
GHB Treated
HOT
2-OH-glutarate
GHB
HOT
2-OH-glutarate
2-hydroxyglutarate
Control
GHB Treated
FA metabolism
FA synthesis
Omega 3 FA
Bile acids
6-beta-hydroxylithocholate
DPA; 22:5n3
DHA; 22:6n3
EPA; 20:5n3
Aminosugars
Glucose and glycolysis
TCA
beta-Ox
Glutathione
Methionine
Homocysteine
S-adenosylhomocysteine (SAH)
GSSG
GSH
Arginine
Glutamate
Histidine
Urea
Purines/Pyrimidines turnover
maltotriose
fructose
citrate
citrate
BHBA
BHBA
5'-AMP
5'-CMP
5'-GMP
xanthosine
allantoin
urea
Liver
Brain

Supplemental Table S1

Increased p<0.05			Decreased p<0.05		Suggestive Change p<0.1
ACUTE					
BRAIN			**LIVER**		
BIOCHEMICAL NAME	*fold change*	*p value*	*BIOCHEMICAL NAME*	*fold change*	*p value*
4-hydroxybutyrate (GHB)	323.20	< 0.001	4-hydroxybutyrate (GHB)	191.67	< 0.001
glycolate (hydroxyacetate)	7.24	< 0.001	2-hydroxyglutarate	28.75	< 0.001
1-docosahexaenoylglycerophosphocholine*	4.15	0.0013	glycolate (hydroxyacetate)	4.96	0.0227
Isobar: fructose 1,6-diphosphate, glucose 1,6-diphosphate	3.54	0.0376	corticosterone	3.85	< 0.001
sphingosine	3.32	0.0028	hexadecanedioate	2.11	0.0107
2-palmitoleoylglycerophosphoethanolamine*	3.22	0.0019	1-methylimidazoleacetate	2.03	< 0.001
1-stearoylglycerophosphocholine	3.22	0.0049	3-(4-hydroxyphenyl)lactate	1.96	< 0.001
stearoylcarnitine	3.21	0.0112	creatinine	1.92	0.0078
1-oleoylglycerophosphocholine	3.09	0.0034	succinyl CoA	1.89	0.0024
1-stearoylglycerophosphoethanolamine	3.06	0.0044	isovalerylglycine	1.74	0.0073
palmitoylcarnitine	3.02	0.0092	glycylproline	1.56	0.0186
2-docosahexaenoylglycerophosphocholine*	2.96	0.0014	choline phosphate	1.40	0.0063
2-myristoylglycerophosphocholine*	2.90	0.0045	adenosine 3'-monophosphate (3'-AMP)	1.34	0.0230
2-palmitoleoylglycerophosphocholine*	2.86	0.0017	dimethylglycine	1.30	0.0281
2-linoleoylglycerophosphocholine*	2.86	0.0022	docosapentaenoate (n6 DPA; 22:5n6)	1.27	0.0433
2-arachidonoylglycerophosphocholine*	2.86	0.0054	hypoxanthine	1.26	0.0455
2-docosapentaenoylglycerophosphoethanolamine*	2.84	0.0096	isoleucine	1.23	0.0213

oleoylcarnitine	2.81	0.0145	valine	1.22	0.0313
2-hydroxyglutarate	2.77	< 0.001	flavin mononucleotide (FMN)	1.15	0.0365
fructose	2.77	0.0011	sucrose	0.20	0.0098
1-linoleoylglycerophosphocholine	2.65	0.0302	tauroursodeoxycholate	0.21	0.0034
2-linoleoylglycerophosphoethanolamine*	2.57	< 0.001	gamma-glutamylthreonine*	0.25	< 0.001
2-palmitoylglycerophosphocholine*	2.53	0.0072	tauro-beta-muricholate	0.40	0.0061
1-palmitoleoylglycerophosphocholine*	2.50	0.0035	taurodeoxycholate	0.41	0.0012
1-myristoylglycerophosphocholine	2.49	< 0.001	taurochenodeoxycholate	0.43	< 0.001
2-oleoylglycerophosphocholine*	2.44	0.0070	cinnamoylglycine	0.44	0.0018
1-palmitoylglycerophosphocholine	2.32	0.0062	taurocholate	0.47	0.0011
2-palmitoylglycerophosphoethanolamine*	2.32	0.0065	6-beta-hydroxylithocholate	0.47	0.0019
2-docosahexaenoylglycerophosphoethanolamine*	2.12	0.0036	alpha-muricholate	0.48	< 0.001
2-arachidonoylglycerophosphoethanolamine*	1.99	0.0096	valerylglycine	0.53	< 0.001
2-oleoylglycerophosphoethanolamine*	1.92	0.0097	beta-muricholate	0.53	0.0057
S-lactoylglutathione	1.83	0.0335	cholate	0.53	0.0356
1-oleoylglycerol (1-monoolein)	1.72	0.0166	ophthalmate	0.54	0.0050
acetyl CoA	1.65	0.0167	sarcosine (N-Methylglycine)	0.57	0.0012
2-palmitoylglycerol (2-monopalmitin)	1.61	0.0074	sorbitol	0.57	0.0013
1-palmitoylglycerol (1-monopalmitin)	1.59	0.0118	2-methylbutyroylcarnitine	0.57	0.0099
1-stearoylglycerol (1-monostearin)	1.48	0.0288	alanylleucine	0.58	0.0095
2-stearoylglycerol (2-monostearin)	1.44	0.0099	gulono-1,4-lactone	0.60	0.0013
beta-alanine	1.42	0.0275	glutamate	0.61	0.0019

pyruvate	1.27	0.0497	xanthosine	0.62	0.0019
valine	1.26	0.0029	N-acetylglutamine	0.65	0.0015
glycerol 2-phosphate	1.21	0.0484	alanine	0.66	< 0.001
N-acetylmethionine	1.18	0.0331	glutamine	0.66	< 0.001
oleate (18:1n9)	1.17	0.0313	1,3-dihydroxyacetone	0.67	0.0016
lactate	1.15	0.0029	5-aminovalerate	0.69	0.0051
cis-vaccenate (18:1n7)	1.15	0.0418	3-phosphoglycerate	0.71	0.0282
aspartate	1.10	0.0473	glycerophosphorylcholine (GPC)	0.72	0.0450
N-acetylaspartate (NAA)	1.05	0.0082	gamma-glutamylvaline	0.73	0.0132
2-aminoadipate	0.59	0.0016	alanylvaline	0.74	0.0312
2-methylbutyroylcarnitine	0.66	0.0046	creatine	0.75	0.0399
alpha-hydroxyisovalerate	0.67	< 0.001	ribitol	0.75	0.0463
isobutyrylcarnitine	0.70	0.0071	glycylleucine	0.78	0.0292
spermidine	0.70	0.0344	ribose	0.78	0.0350
caproate (6:0)	0.72	0.0029	xylitol	0.79	0.0121
propionylcarnitine	0.73	0.0124	lactate	0.81	0.0169
isovalerylcarnitine	0.75	0.0406	beta-hydroxypyruvate	0.81	0.0273
3-hydroxybutyrate (BHBA)	0.77	0.0116	myo-inositol	0.83	0.0451
butyrylcarnitine	0.78	0.0351	gamma-glutamylleucine	0.84	0.0085
malate	0.80	0.0027	glycerol	0.89	0.0413
gamma-glutamylglutamate	0.81	< 0.001	glucose	0.90	0.0070
5-methylthioadenosine (MTA)	0.82	0.0026	cytidine	**1.63**	0.0534
citrate	0.84	0.0453	betaine aldehyde	**1.49**	0.0928
glutamate	0.85	0.0028	homoserine	**1.37**	0.0584
nicotinamide	0.85	0.0128	adenosine 2'-monophosphate (2'-AMP)	**1.33**	0.0781
carnitine	0.87	0.0213	imidazole lactate	**1.27**	0.0548
choline phosphate	0.89	0.0099	lysine	**1.23**	0.0586
2-stearoylglycerophosphocholine*	**2.65**	0.0569	riboflavin (Vitamin B2)	**1.23**	0.0903
alpha-tocopherol	**1.50**	0.0543	citrulline	**1.21**	0.0938

docosapentaenoate (n6 DPA; 22:5n6)	**1.33**	0.0741	tryptophan	**1.19**	0.0659
Isobar: ribulose 5-phosphate, xylulose 5-phosphate	**1.25**	0.0558	inosine 5'-monophosphate (IMP)	**0.24**	0.0945
1,3-dipalmitoylglycerol	**1.25**	0.0861	2-aminoadipate	**0.64**	0.0535
prostaglandin E2	**1.24**	0.0928	acetylcarnitine	**0.64**	0.0756
1,2-dipalmitoylglycerol	**1.23**	0.0979	1-linoleoylglycerol (1-monolinolein)	**0.64**	0.0821
tryptophan	**1.22**	0.0910	phosphoenolpyruvate (PEP)	**0.71**	0.0858
docosahexaenoate (DHA; 22:6n3)	**1.15**	0.0838	glycerate	**0.75**	0.0575
leucine	**1.13**	0.0889	glycerol 2-phosphate	**0.75**	0.0938
cholesterol	**1.12**	0.0513	3-aminoisobutyrate	**0.81**	0.0620
caprylate (8:0)	**0.85**	0.0797	2-aminobutyrate	**0.83**	0.0822
10-heptadecenoate (17:1n7)	**0.86**	0.0775	trans-4-hydroxyproline	**0.84**	0.0873
taurine	**0.87**	0.0513	5-oxoproline	**0.87**	0.0992
N-acetylglutamate	**0.92**	0.0528	methylglutaroylcarnitine	1.24	0.1005
S-adenosylhomocysteine (SAH)	0.90	0.1102	cysteine-glutathione disulfide	1.33	0.1009
phosphoethanolamine	0.95	0.1123	xanthine	1.15	0.1021
glucose	1.71	0.1134	glutarate (pentanedioate)	1.30	0.1028
pipecolate	0.87	0.1146	ribulose	0.84	0.1057
sorbitol	1.38	0.1164	3-hydroxybutyrate (BHBA)	0.77	0.1090
uridine	1.11	0.1175	12,13-hydroxyoctadec-9(Z)-enoate	0.75	0.1095
4-guanidinobutanoate	0.91	0.1228	pyridoxal	1.36	0.1208
C-glycosyltryptophan*	0.92	0.1248	leucine	1.11	0.1223
adenosine 2'-monophosphate (2'-AMP)	1.18	0.1269	flavin adenine dinucleotide (FAD)	1.10	0.1268
2-phosphoglycerate	1.93	0.1274	thiamin (Vitamin B1)	0.90	0.1348
1-palmitoylglycerophosphoethanolamine	1.20	0.1313	threonate	0.72	0.1349
prostaglandin D2	1.29	0.1389	pyridoxate	1.33	0.1353
glycerol 3-phosphate (G3P)	1.19	0.1407	guanosine	1.34	0.1386
3-phosphoglycerate	1.58	0.1417	gluconate	0.87	0.1390
sarcosine (N-Methylglycine)	1.13	0.1506	cysteinylglycine	0.74	0.1395

isoleucine	1.09	0.1533	adrenate (22:4n6)	1.18	0.1422
glycylleucine	0.72	0.1577	1-pentadecanoylglycerophosphocholine*	1.17	0.1466
palmitoleate (16:1n7)	0.90	0.1686	phenylalanine	1.13	0.1467
5-aminovalerate	0.79	0.1690	coenzyme A	0.76	0.1469
pseudouridine	0.93	0.1732	luteolin-7-O-glucoside	0.82	0.1517
glutathione, oxidized (GSSG)	0.90	0.1927	1,5-anhydroglucitol (1,5-AG)	0.81	0.1631
creatinine	0.93	0.1940	beta-alanine	0.74	0.1684
coenzyme A	1.41	0.1961	hexanoylglycine	0.83	0.1696
glycine	1.08	0.1999	hydroxyisovaleroyl carnitine	1.16	0.1731
1-arachidonoylglycerophosphoinositol*	1.19	0.2076	kynurenine	1.23	0.1828
N-acetylneuraminate	1.09	0.2097	alpha-tocopherol	1.22	0.1853
acetylphosphate	0.87	0.2139	glycylvaline	1.11	0.1918
creatine	0.95	0.2165	cytidine 5'-monophosphate (5'-CMP)	1.15	0.1964
arginine	0.94	0.2213	Isobar: fructose 1,6-diphosphate, glucose 1,6-diphosphate	0.47	0.1966
gamma-aminobutyrate (GABA)	0.93	0.2234	5-methylthioadenosine (MTA)	0.79	0.2064
sedoheptulose-7-phosphate	1.35	0.2372	malate	0.91	0.2092
3'-dephosphocoenzyme A	1.52	0.2407	nonadecanoate (19:0)	1.15	0.2123
N-acetylalanine	0.88	0.2446	ethyl glucuronide	0.47	0.2143
serine	0.95	0.2475	docosahexaenoate (DHA; 22:6n3)	1.14	0.2162
ribose 5-phosphate	1.18	0.2483	12-dehydrocholate	0.67	0.2175
glutamine	1.15	0.2704	glucuronate	1.07	0.2242
1-oleoylglycerophosphoethanolamine	1.16	0.2802	1-arachidonoylglycerophosphoinositol*	1.20	0.2281
homocarnosine	0.92	0.2888	dimethylarginine (SDMA + ADMA)	1.21	0.2300
acetylcarnitine	0.86	0.3050	malonylcarnitine	0.87	0.2365
palmitoyl sphingomyelin	1.08	0.3125	glycine	1.08	0.2367
7-alpha-hydroxycholesterol	1.14	0.3221	N-acetylasparagine	0.87	0.2372

arabitol	1.14	0.3276	myristoleate (14:1n5)	1.08	0.2372
proline	1.07	0.3361	arachidonate (20:4n6)	1.13	0.2381
cytidine 5'-monophosphate (5'-CMP)	0.89	0.3454	3'-dephosphocoenzyme A	0.90	0.2382
glycerol	0.97	0.3548	sphingosine	1.11	0.2395
pyrophosphate (PPi)	0.85	0.4002	5-methyltetrahydrofolate (5MeTHF)	1.36	0.2502
glutaroyl carnitine	0.92	0.4018	fructose	0.91	0.2514
mannose-6-phosphate	1.33	0.4063	1-heptadecanoylglycerophosphocholine	1.20	0.2593
adenine	0.94	0.4231	biopterin	1.17	0.2606
arabinose	1.11	0.4236	pyroglutamine*	1.15	0.2607
urea	0.96	0.4267	1-palmitoleoylglycerophosphocholine*	0.89	0.2645
1-methylimidazoleacetate	0.91	0.4407	methionine	1.12	0.2682
adenosine	0.86	0.4448	adenosine 5'diphosphoribose	0.37	0.2693
pantothenate	0.96	0.4466	threonine	1.08	0.2706
dihomo-linoleate (20:2n6)	1.12	0.4481	glutathione, reduced (GSH)	0.89	0.2717
guanosine	0.90	0.4503	6-phosphogluconate	1.14	0.2815
N-acetyl-aspartyl-glutamate (NAAG)	1.06	0.4507	N-acetylglutamate	1.07	0.2921
linoleate (18:2n6)	1.11	0.4510	acetylphosphate	0.86	0.2969
stearoyl sphingomyelin	1.07	0.4556	spermidine	0.92	0.2996
1-oleoylglycerophosphoserine	1.11	0.4644	allantoin	0.78	0.2998
argininosuccinate	0.96	0.4645	uridine	1.09	0.3015
erythronate*	1.04	0.4737	1-stearoylglycerophosphocholine	0.86	0.3059
ribose	1.11	0.4809	2-arachidonoylglycerophosphocholine*	0.93	0.3181
5-HETE	0.95	0.4823	uracil	1.15	0.3331
myo-inositol	0.95	0.4846	1-docosahexaenoylglycerophosphocholine*	1.18	0.3349
scyllo-inositol	0.92	0.4921	1-oleoylglycerophosphoinositol*	1.17	0.3406

glycerate	1.07	0.4939	glycylglycine	1.13	0.3411
1-stearoylglycerophosphoinositol	1.07	0.4944	caprylate (8:0)	0.88	0.3546
mead acid (20:3n9)	1.09	0.4989	2-arachidonoylglycerophosphoinositol*	1.14	0.3587
inositol 2-phosphate (I2P)	1.08	0.5102	dihomo-linolenate (20:3n3 or n6)	1.09	0.3595
lysine	0.95	0.5126	myristate (14:0)	1.04	0.3627
1-palmitoylglycerophosphoinositol*	1.07	0.5134	adenine	1.23	0.3667
carnosine	1.04	0.5164	taurine	1.09	0.3697
flavin adenine dinucleotide (FAD)	1.01	0.5255	2-palmitoleoylglycerophosphocholine*	0.85	0.3707
ascorbate (Vitamin C)	0.92	0.5287	2-oleoylglycerol (2-monoolein)	0.81	0.3711
phenylalanine	1.06	0.5449	2-docosahexaenoylglycerophosphocholine*	0.94	0.3747
methionine	0.97	0.5455	glutathione, oxidized (GSSG)	1.08	0.3831
fumarate	0.82	0.5462	serine	1.07	0.3856
2-pyrrolidinone	1.16	0.5595	maltohexaose	1.17	0.3938
1-oleoylglycerophosphoinositol*	1.05	0.5673	Isobar: ribulose 5-phosphate, xylulose 5-phosphate	0.83	0.3938
inosine	1.03	0.5736	laurate (12:0)	1.05	0.4017
deoxycarnitine	0.94	0.5772	N-glycolylneuraminate	0.91	0.4041
myristate (14:0)	0.99	0.5989	butyrylglycine	0.89	0.4049
13-HODE + 9-HODE	0.92	0.5991	maltopentaose	1.12	0.4104
gamma-glutamylleucine	1.06	0.6105	palmitoyl ethanolamide	0.73	0.4199
N-acetylglutamine	1.00	0.6159	glycylphenylalanine	0.87	0.4210
adrenate (22:4n6)	1.13	0.6234	glycerol 3-phosphate (G3P)	0.90	0.4229
ophthalmate	0.96	0.6276	methyl palmitate (15 or 2)	1.05	0.4259
3-phosphoserine	1.92	0.6330	propionylcarnitine	1.35	0.4312
1-palmitoylplasmenylethanolamine*	1.05	0.6338	erythronate*	0.92	0.4347
threonine	1.04	0.6373	2-linoleoylglycerophospho	0.96	0.4399

			choline*		
stearate (18:0)	0.98	0.6382	1-eicosatrienoylglycerophosphocholine*	0.91	0.4400
anserine	1.05	0.6408	carnitine	0.95	0.4496
3-(4-hydroxyphenyl)lactate	1.00	0.6480	phenylacetylglycine	0.82	0.4516
ergothioneine	0.95	0.6550	urate	1.34	0.4522
phosphate	1.01	0.6607	n-Butyl Oleate	0.91	0.4538
myristoleate (14:1n5)	0.96	0.6645	tagatose	1.33	0.4544
gamma-glutamylglutamine	1.04	0.6777	adenosine 5'-monophosphate (AMP)	0.75	0.4553
tyrosine	1.09	0.6850	10-nonadecenoate (19:1n9)	1.09	0.4566
pyroglutamine*	1.08	0.6854	3-indoxyl sulfate	0.91	0.4591
1-arachidonoylglycerophosphoethanolamine*	0.94	0.6908	nicotinamide	0.95	0.4597
xanthine	1.04	0.7007	1-stearoylglycerophosphoinositol	1.17	0.4630
cysteine	1.09	0.7102	phosphoethanolamine	0.91	0.4651
hypoxanthine	1.03	0.7121	4-guanidinobutanoate	1.16	0.4657
margarate (17:0)	1.07	0.7165	1-arachidonoylglycerophosphoethanolamine*	1.28	0.4708
arachidonate (20:4n6)	1.04	0.7327	proline	1.06	0.4800
palmitate (16:0)	1.03	0.7448	1,3-dipalmitoylglycerol	1.07	0.4800
glucose-6-phosphate (G6P)	1.26	0.7513	palmitate (16:0)	1.06	0.4872
urate	1.12	0.7555	choline	1.07	0.4885
pelargonate (9:0)	0.99	0.7567	pro-hydroxy-pro	0.89	0.4906
laurate (12:0)	1.01	0.7752	succinylcarnitine	0.87	0.4926
methylphosphate	1.04	0.7753	thymidine	1.18	0.4947
3-dehydrocarnitine*	0.97	0.7826	1-palmitoylglycerophosphoinositol*	1.16	0.4997
trans-4-hydroxyproline	1.08	0.7830	mannose-6-phosphate	1.17	0.5008
2-hydroxybutyrate (AHB)	0.97	0.7849	1-arachidoylglycerophosphocholine	0.81	0.5052
adenosine 5'-monophosphate (AMP)	0.84	0.7866	cytidine 5'-diphosphocholine	0.84	0.5155

chiro-inositol	1.00	0.8113	dihomo-linoleate (20:2n6)	1.07	0.5206
allo-threonine	1.01	0.8188	homostachydrine*	0.89	0.5217
homoserine	1.02	0.8260	methylphosphate	0.80	0.5287
caprate (10:0)	0.99	0.8337	nicotinamide adenine dinucleotide (NAD+)	1.05	0.5323
glutamate, gamma-methyl ester	1.00	0.8415	maltotriose	0.94	0.5340
dehydroascorbate	1.05	0.8586	urea	0.96	0.5380
docosapentaenoate (n3 DPA; 22:5n3)	1.08	0.8589	2-hydroxybutyrate (AHB)	1.13	0.5385
nicotinamide adenine dinucleotide (NAD+)	1.02	0.8607	glutaroyl carnitine	1.20	0.5387
hydroxyisovaleroyl carnitine	0.99	0.8633	7,8-dihydrofolate	1.08	0.5389
allantoin	0.94	0.8674	isobutyrylcarnitine	0.84	0.5409
mannitol	1.02	0.8703	glutamate, gamma-methyl ester	0.75	0.5477
dihomo-linolenate (20:3n3 or n6)	0.99	0.8809	pipecolate	1.06	0.5497
7-beta-hydroxycholesterol	1.01	0.8817	C-glycosyltryptophan*	1.05	0.5511
cytidine	1.01	0.9030	stearoyl sphingomyelin	0.95	0.5602
fructose-6-phosphate	1.14	0.9091	2-docosahexaenoylglycerophosphoethanolamine*	1.05	0.5606
linolenate [alpha or gamma; (18:3n3 or 6)]	0.99	0.9165	nicotinamide adenine dinucleotide reduced (NADH)	1.09	0.5631
10-nonadecenoate (19:1n9)	1.01	0.9208	aspartate	0.97	0.5644
inositol 1-phosphate (I1P)	0.99	0.9306	docosatrienoate (22:3n3)	1.11	0.5649
24(S)-hydroxycholesterol	0.98	0.9314	1-eicosadienoylglycerophosphocholine*	1.12	0.5750
1,5-anhydroglucitol (1,5-AG)	1.02	0.9358	S-methylglutathione	1.11	0.5823
eicosenoate (20:1n9 or 11)	1.02	0.9370	2-phosphoglycerate	0.88	0.5844
alanine	1.00	0.9403	tetradecanedioate	1.16	0.5873
uracil	1.01	0.9495	1-linoleoylglycerophosphocholine	0.94	0.5897
5-oxoproline	1.02	0.9615	1-linoleoylglycerophosphoethanolamine*	1.09	0.5912
ethanolamine	1.02	0.9687	asparagine	0.91	0.5918

glycerophosphorylcholine (GPC)	1.05	0.9739	hypotaurine	1.09	0.5961
N-formylmethionine	1.01	0.9746	phosphate	1.02	0.5988
cysteine-glutathione disulfide	1.02	0.9774	dihydroxyacetone phosphate (DHAP)	0.80	0.6021
glutathione, reduced (GSH)	1.09	0.9989	heme*	1.07	0.6076
			azelate (nonanedioate)	0.92	0.6079
			gamma-glutamylglutamate	0.97	0.6122
			2-linoleoylglycerophosphoethanolamine*	1.04	0.6130
			docosadienoate (22:2n6)	1.07	0.6202
			eicosapentaenoate (EPA; 20:5n3)	0.96	0.6257
			stearate (18:0)	1.06	0.6265
			3-dehydrocarnitine*	1.12	0.6276
			5-methylcytidine	0.82	0.6342
			2-arachidonoylglycerophosphoethanolamine*	1.03	0.6436
			1-palmitoylglycerol (1-monopalmitin)	1.18	0.6462
			guanosine 5'-monophosphate (5'-GMP)	0.73	0.6480
			margarate (17:0)	1.03	0.6529
			S-adenosylhomocysteine (SAH)	1.06	0.6610
			uridine monophosphate (5' or 3')	0.75	0.6728
			ribose 5-phosphate	0.94	0.6732
			xylonate	0.97	0.6790
			ethanolamine	1.05	0.6790
			N-acetylmethionine	0.96	0.6803
			cholesterol	1.03	0.6830
			1-oleoylglycerophosphocholine	0.97	0.6836
			13-HODE + 9-HODE	0.97	0.6890
			1-palmitoylglycerophospho	0.99	0.6905

choline		
N-acetylneuraminate	0.96	0.6979
Isobar: 2-propylpentanoic acid, 2-ethylhexanoic acid	1.02	0.7054
2-oleoylglycerophosphocholine*	0.97	0.7064
10-heptadecenoate (17:1n7)	1.02	0.7146
cysteine	0.97	0.7161
7-alpha-hydroxycholesterol	1.05	0.7292
glycyltyrosine	0.96	0.7300
7-beta-hydroxycholesterol	1.00	0.7336
2-oleoylglycerophosphoethanolamine*	1.03	0.7348
pantothenate	0.97	0.7353
1-myristoylglycerophosphocholine	0.97	0.7367
methionine sulfoxide	0.99	0.7382
N-acetylleucine	0.96	0.7497
16-hydroxypalmitate	1.02	0.7659
adenosine	0.96	0.7664
N-acetylalanine	1.05	0.7680
eicosenoate (20:1n9 or 11)	0.94	0.7695
glucose-6-phosphate (G6P)	0.92	0.7778
fumarate	0.98	0.7847
ergothioneine	1.05	0.7853
mannose	1.01	0.7934
N-acetylornithine	1.00	0.7934
fructose-6-phosphate	0.97	0.8017
caproate (6:0)	1.01	0.8063
2-stearoylglycerophosphocholine*	0.87	0.8100

phosphopantetheine	1.03	0.8165
linoleate (18:2n6)	1.03	0.8201
maltose	0.95	0.8221
palmitoleate (16:1n7)	0.97	0.8340
stachydrine	1.01	0.8371
1-stearoylglycerophosphoethanolamine	1.07	0.8375
tyrosine	0.99	0.8384
1-stearoylglycerol (1-monostearin)	1.11	0.8394
inositol 1-phosphate (I1P)	1.03	0.8404
docosapentaenoate (n3 DPA; 22:5n3)	0.99	0.8498
17-methylstearate	0.95	0.8541
2-palmitoylglycerophosphocholine*	0.98	0.8633
S-lactoylglutathione	1.12	0.8696
inosine	0.99	0.8713
dihydrobiopterin	0.97	0.8761
pyruvate	0.88	0.8825
caprate (10:0)	0.99	0.8829
palmitoyl sphingomyelin	1.00	0.8847
oleate (18:1n9)	1.02	0.8850
2-palmitoylglycerophosphoethanolamine*	1.01	0.8891
1-arachidonoylglycerophosphocholine*	1.00	0.8912
2-docosapentaenoylglycerophosphoethanolamine*	1.03	0.8921
1-oleoylglycerophosphoethanolamine	1.03	0.9029
15-HETE	1.09	0.9046
1-palmitoylglycerophosphoethanolamine	1.01	0.9061

betaine	1.12	0.9198
ornithine	0.99	0.9202
ascorbate (Vitamin C)	0.99	0.9209
deoxycarnitine	1.07	0.9330
glycylisoleucine	1.01	0.9349
campesterol	0.99	0.9360
stearidonate (18:4n3)	1.04	0.9493
UDP-galactose	0.91	0.9502
histidine	1.01	0.9536
maltotetraose	1.00	0.9596
linolenate [alpha or gamma; (18:3n3 or 6)]	1.01	0.9626
citrate	1.01	0.9666
1,2-dipalmitoylglycerol	0.87	0.9856
pyrophosphate (PPi)	0.91	0.9881
adenosine 3',5'-diphosphate	0.95	0.9885
3-ureidopropionate	1.00	1.00

Supplemental Table S2

Genotype/Week	**Treated** (Mean ± SD)	**Control** (Mean ± SD)	**"treatment"**	**"week"**	**"treatment x week"**
C57BL/6J (N=20)					
Initial weight	25.54 ± 1.31	25.62 ± 1.18			
Week 1	24.52 ± 1.46	25.04 ± 1.04			
Week 2	25.86 ± 1.25	26.77 ± 1.67			
Week 3	25.86 ± 0.92	27.02 ± 1.40			
Week 4	26.53 ± 0.90	27.40 ± 1.36	0.001	0.002	0.271
Ob/Ob (N=16)					
Initial weight	46.03 ± 3.74	46.50 ± 1.65			
Week 1	44.55 ± 6.13	46.11 ± 1.44			
Week 2	46.72 ± 7.58	48.12 ± 1.69			
Week 3	51.73 ± 8.00	52.85 ± 2.38			
Week 4	51.44 ± 7.73	53.16 ± 1.68	0.596	<0.001	0.783
GABA-B kO (N=11)					
Initial weight	27.00 ± 0.95	27.12 ± 1.03			
Week 1	28.30 ± 1.68	28.00 ± 1.59			
Week 2	28.00 ± 1.74	28.70 ± 1.41			
Week 3	27.40 ± 1.34	29.50 ± 2.13	0.002	0.196	<0.001

Weight changes in GHB treated and control C57BL/6J, Ob/Ob and GABA-B kO mice. Effects of treatment, week and their interaction are represented by p values of two-way RM ANOVA.

Supplementary Table 3

CHRONIC					
BRAIN			LIVER		
BIOCHEMICAL NAME	*fold change*	*p value*	*BIOCHEMICAL NAME*	*fold change*	*p value*
inosine 5'-monophosphate (IMP)	2.83	0.0440	cytidine-3'-monophosphate (3'-CMP)	21.85	0.0005
guanosine 5'-monophosphate (5'-GMP)	1.77	0.0429	2-hydroxyglutarate	5.83	0.0050
2-linoleoylglycerophosphocholine*	1.45	0.0134	6-beta-hydroxylithocholate	1.47	0.0278
1-linoleoylglycerophosphocholine	1.34	0.0176	leucylalanine	1.35	0.0471
Isobar: UDP-acetylglucosamine, UDP-acetylgalactosamine	1.23	0.0186	maltotriose	1.31	0.0140
cytidine 5'-monophosphate (5'-CMP)	1.14	0.0234	tetradecanedioate	1.29	0.0497
citrate	1.12	0.0151	kynurenine	1.27	0.0252
nicotinate	1.10	0.0303	glycerate	1.25	0.0340
allantoin	0.53	0.0012	N-acetylalanine	1.21	0.0451
2-hydroxypalmitate	0.71	0.0147	docosahexaenoate (DHA; 22:6n3)	1.19	0.0231
3-hydroxybutyrate (BHBA)	0.72	0.0099	palmitoyl sphingomyelin	1.16	0.0152
urea	0.78	0.0008	gamma-glutamylglutamate	1.07	0.0251
xanthosine	0.78	0.0133	1-heptadecanoylglycerophosphocholine	0.42	0.0265
1-methylimidazoleacetate	0.80	0.0092	1-stearoylglycerophosphocholine	0.47	0.0434
1-stearoylglycerophosphoinositol	0.80	0.0288	2-oleoylglycerophosphocholine*	0.55	0.0062
1-arachidonoylglycerophosphoinositol*	0.82	0.0370	1-eicosatrienoylglycerophosphocholine*	0.59	0.0027
adenine	0.85	0.0311	fructose	0.60	0.0148
S-adenosylhomocysteine (SAH)	0.87	0.0049	1-arachidonoylglycerophosphocholine*	0.63	0.0109
glutathione, oxidized (GSSG)	0.92	0.0098	2-eicosatrienoylglycerophosphocholine*	0.63	0.0073

myo-inositol	0.93	0.0408	sphingosine	0.63	0.0334
4-hydroxy-2-nonenal	1.29	0.0827	2-palmitoylglycerophosphocholine*	0.63	0.0251
2-palmitoylglycerophosphocholine*	1.22	0.0893	2-docosahexaenoylglycerophosphocholine*	0.64	0.0017
15-HETE	1.14	0.0861	1-linoleoylglycerophosphocholine	0.64	0.0194
2-palmitoylglycerol (2-monopalmitin)	1.13	0.0582	2-linoleoylglycerophosphocholine*	0.65	0.0003
N-acetylmethionine	1.10	0.0843	mannose	0.66	0.0178
2-hydroxystearate	0.70	0.0782	1-eicosadienoylglycerophosphocholine*	0.66	0.0051
2-pyrrolidinone	0.70	0.0698	1-oleoylglycerophosphocholine	0.68	0.0276
arabitol	0.77	0.0709	4-guanidinobutanoate	0.68	0.0008
nicotinamide adenine dinucleotide (NAD+)	0.81	0.0536	1-palmitoylglycerophosphocholine	0.69	0.0045
6-keto prostaglandin F1alpha	0.81	0.0882	gamma-glutamylthreonine*	0.70	0.0448
2-stearoylglycerophosphoinositol*	0.83	0.0661	methylphosphate	0.71	0.0112
1,5-anhydroglucitol (1,5-AG)	0.83	0.0638	2-arachidonoylglycerophosphocholine*	0.71	0.0084
riboflavin (Vitamin B2)	0.84	0.0758	2-docosapentaenoylglycerophosphocholine*	0.73	0.0266
N1-methyladenosine	0.85	0.0592	trans-4-hydroxyproline	0.73	0.0428
glucose-6-phosphate (G6P)	0.85	0.0865	2-arachidonoylglycerophosphoethanolamine*	0.74	0.0016
erythritol	0.86	0.0798	2-docosahexaenoylglycerophosphoethanolamine*	0.74	0.0078
xanthine	0.88	0.0734	phosphopantetheine	0.75	0.0050
propionylcarnitine	0.91	0.0727	2-palmitoleoylglycerophosphocholine*	0.75	0.0147
threonine	0.92	0.0613	isovalerylglycine	0.77	0.0136

serine	0.93	0.0587	sedoheptulose-7-phosphate	0.77	0.0319
nicotinamide	0.93	0.0678	urea	0.81	0.0155
1-palmitoylglycerophosphoinositol*	0.87	0.1007	trigonelline (N'-methylnicotinate)	0.82	0.0182
mannose-6-phosphate	0.74	0.1013	4-hydroxybutyrate (GHB)	1.63	0.0521
creatine	1.04	0.1078	alpha-muricholate	1.54	0.0747
spermine	0.62	0.1091	docosadienoate (22:2n6)	1.53	0.0776
oleate (18:1n9)	0.93	0.1109	tagatose	1.50	0.0754
adenosine 2'-monophosphate (2'-AMP)	1.19	0.1123	1-palmitoylglycerol (1-monopalmitin)	1.44	0.0877
1-arachidonoylglycerophosphoethanolamine*	0.79	0.1172	taurodeoxycholate	1.42	0.0769
prostaglandin E2	1.10	0.1203	stearidonate (18:4n3)	1.37	0.0919
adenylosuccinate	1.49	0.1213	12,13-hydroxyoctadec-9(Z)-enoate	1.35	0.0762
catechol sulfate	0.77	0.1262	leucylthreonine	1.34	0.0866
1-palmitoylglycerophosphocholine	1.16	0.1277	docosapentaenoate (n3 DPA; 22:5n3)	1.32	0.0503
sorbitol	0.85	0.1309	alpha-hydroxyisovalerate	1.29	0.0650
2-methylbutyrylcarnitine (C5)	0.82	0.1343	serylleucine	1.26	0.0641
cytidine-5'-diphosphoethanolamine	1.04	0.1343	eicosapentaenoate (EPA; 20:5n3)	1.25	0.0596
acetylcarnitine	0.95	0.1399	homostachydrine*	1.24	0.0575
guanosine 5'-diphospho-fucose	0.93	0.1415	isoleucylalanine	1.20	0.0557
palmitoylcarnitine	1.23	0.1420	uracil	1.17	0.0843
2-docosahexaenoylglycerophosphocholine*	1.22	0.1422	inositol 1-phosphate (I1P)	1.16	0.0553
adenosine 5'-monophosphate (AMP)	1.38	0.1471	lysine	1.08	0.0506
oleoylcarnitine	1.26	0.1474	1-arachidoylglycerophosphocholine	0.42	0.0629
stearate (18:0)	0.95	0.1502	gamma-glutamylglycine	0.66	0.0722
butyrylcarnitine	0.86	0.1530	sphinganine	0.69	0.0860

1-stearoylglycerophosphoethanolamine	0.77	0.1557	ribitol	0.77	0.0511
carnitine	0.92	0.1641	N-acetylleucine	0.78	0.0793
pyroglutamine*	1.07	0.1647	2-linoleoylglycerophosphoethanolamine*	0.79	0.0858
homocarnosine	1.13	0.1688	hydroxybutyrylcarnitine*	0.79	0.0649
1-docosahexaenoylglycerophosphocholine*	1.21	0.1758	N-acetylglutamate	0.82	0.0913
3-dehydrocarnitine*	0.94	0.1800	N-acetylmethionine	0.85	0.0513
choline phosphate	0.95	0.1807	uridine	0.86	0.0577
deoxycarnitine	1.08	0.1898	1-palmitoleoylglycerophosphocholine*	0.82	0.1030
cholesterol	0.92	0.1934	glycylisoleucine	1.29	0.1039
gamma-aminobutyrate (GABA)	0.93	0.1941	tauroursodeoxycholate	1.50	0.1041
pyrophosphate (PPi)	0.89	0.1949	taurine	1.36	0.1047
N-acetyl-aspartyl-glutamate (NAAG)	0.89	0.1982	erythritol	1.29	0.1059
1,2-dipalmitoylglycerol	0.82	0.2101	S-adenosylhomocysteine (SAH)	0.91	0.1084
stearoylcarnitine	1.16	0.2204	tauro(alpha + beta)muricholate	1.46	0.1117
5-HETE	1.10	0.2223	adenine	1.21	0.1119
glycine	0.95	0.2295	adrenate (22:4n6)	1.29	0.1131
glycolate (hydroxyacetate)	0.89	0.2347	dihomo-linoleate (20:2n6)	1.35	0.1152
thymidine	0.92	0.2475	cis-vaccenate (18:1n7)	1.62	0.1154
uridine	1.03	0.2526	10-heptadecenoate (17:1n7)	1.26	0.1166
10-heptadecenoate (17:1n7)	1.08	0.2540	1-linoleoylglycerophosphoethanolamine*	0.84	0.1172
guanosine	1.07	0.2547	1-docosapentaenoylglycerophosphocholine*	0.83	0.1173
adenosine 5'diphosphoribose	0.44	0.2562	cysteine	1.58	0.1211
2-arachidonoylglycerophosphocholine*	1.17	0.2593	xylitol	0.76	0.1252

7-alpha-hydroxycholesterol	1.15	0.2598	1-docosahexaenoylglycerophosphocholine*	0.81	0.1262
24(S)-hydroxycholesterol	0.92	0.2602	gamma-glutamylleucine	0.89	0.1276
proline	0.96	0.2607	glycylvaline	1.22	0.1290
4-guanidinobutanoate	1.10	0.2755	dihomo-linolenate (20:3n3 or n6)	1.14	0.1312
choline	0.94	0.2900	oleate (18:1n9)	1.62	0.1313
lactate	1.03	0.2903	choline phosphate	0.79	0.1371
valine	0.96	0.2922	thymidine	0.84	0.1372
glutathione, reduced (GSH)	1.18	0.2924	eicosenoate (20:1n9 or 11)	1.45	0.1385
2-docosahexaenoylglycerophosphoethanolamine*	1.09	0.2979	maltose	2.26	0.1390
ergothioneine	1.07	0.2979	dimethylglycine	0.86	0.1394
pseudouridine	0.95	0.3058	linolenate [alpha or gamma; (18:3n3 or 6)]	1.22	0.1395
scyllo-inositol	0.93	0.3144	choline	0.93	0.1458
glutarylcarnitine (C5)	0.94	0.3311	acetyl CoA	0.87	0.1461
1-oleoylglycerophosphoethanolamine	0.78	0.3351	glycerol	1.40	0.1471
glycerol 3-phosphate (G3P)	0.94	0.3356	leucine	1.08	0.1471
2-arachidonoylglycerophosphoinositol*	0.92	0.3367	cytidine 5'-diphosphocholine	0.84	0.1473
alpha-tocopherol	0.80	0.3430	3-hydroxybutyrate (BHBA)	0.83	0.1479
alanine	0.97	0.3491	docosapentaenoate (n6 DPA; 22:5n6)	1.40	0.1480
2-palmitoylglycerophosphoethanolamine*	1.13	0.3535	inosine	0.94	0.1498
docosapentaenoate (n3 DPA; 22:5n3)	1.08	0.3574	adenosine 3'-monophosphate (3'-AMP)	1.97	0.1536
homoserine	0.91	0.3574	1,5-anhydroglucitol (1,5-AG)	0.79	0.1540
methylphosphate	0.93	0.3581	2-palmitoylglycerol (2-monopalmitin)	1.63	0.1552
3'-dephosphocoenzyme A	1.20	0.3582	hexadecanedioate	2.57	0.1572
1-stearoylglycerol (1-monostearin)	0.91	0.3613	gamma-glutamylglutamine	3.30	0.1589

cysteine-glutathione disulfide	0.92	0.3636	catechol sulfate	0.80	0.1598
N-acetylneuraminate	0.95	0.3801	adenosine	0.91	0.1601
inositol 1-phosphate (I1P)	1.07	0.3849	10-nonadecenoate (19:1n9)	1.45	0.1606
phenylalanine	0.96	0.3851	cholate	1.61	0.1636
1-oleoylglycerophosphocholine	1.13	0.3874	sarcosine (N-Methylglycine)	0.84	0.1637
N-formylmethionine	1.05	0.3897	nonadecanoate (19:0)	1.26	0.1643
glycerol	0.97	0.3944	histidine	0.86	0.1680
tyrosine	0.96	0.4033	valerylglycine	0.86	0.1684
homostachydrine*	1.02	0.4042	xanthosine	1.12	0.1701
caproate (6:0)	0.93	0.4057	16-hydroxypalmitate	2.46	0.1709
Isobar: ribulose 5-phosphate, xylulose 5-phosphate	1.08	0.4093	1-linoleoylglycerol (1-monolinolein)	1.20	0.1721
prostaglandin D2	1.07	0.4149	N-acetylglutamine	0.91	0.1750
3-(4-hydroxyphenyl)lactate	0.95	0.4274	docosatrienoate (22:3n3)	1.70	0.1757
2-oleoylglycerophosphoethanolamine*	0.86	0.4414	succinate	1.24	0.1794
docosahexaenoate (DHA; 22:6n3)	1.03	0.4507	taurocholate	1.38	0.1799
N-acetylaspartate (NAA)	1.02	0.4584	5-methyltetrahydrofolate (5MeTHF)	0.77	0.1817
hypotaurine	1.07	0.4626	2-aminobutyrate	0.82	0.1828
uracil	0.95	0.4648	margarate (17:0)	1.21	0.1845
glucose	1.26	0.4655	ophthalmate	0.79	0.1941
2-arachidonoylglycerophosphoethanolamine*	0.91	0.4672	xanthine	0.90	0.1949
cytidine	0.95	0.4686	1-myristoylglycerophosphocholine	0.81	0.1953
citrulline	0.97	0.4751	citrate	1.20	0.1960
1-palmitoylplasmenylethanolamine*	0.80	0.4777	linoleate (18:2n6)	1.14	0.1981
methionine	0.97	0.4902	taurochenodeoxycholate	1.28	0.2024
acetyl CoA	1.12	0.5039	proline	0.94	0.2051

1-palmitoylglycerophosphoethanolamine	0.83	0.5119	palmitoleate (16:1n7)	1.24	0.2096
palmitate (16:0)	0.97	0.5204	cinnamoylglycine	0.85	0.2103
dihomo-linoleate (20:2n6)	1.05	0.5238	17-methylstearate	1.21	0.2138
1-oleoylglycerophosphoserine	1.09	0.5320	12-HETE	0.85	0.2164
coenzyme A	0.97	0.5348	taurolithocholate	1.22	0.2181
glutamate	1.02	0.5501	gulono-1,4-lactone	0.86	0.2190
trans-urocanate	0.91	0.5514	glycylphenylalanine	1.10	0.2224
malate	0.98	0.5810	arachidonate (20:4n6)	1.10	0.2240
margarate (17:0)	1.03	0.5878	flavin mononucleotide (FMN)	0.93	0.2288
2'-deoxycytidine	1.05	0.5891	myo-inositol	0.93	0.2297
isoleucine	0.97	0.5975	ribulose	0.83	0.2302
fumarate	0.97	0.6114	15-methylpalmitate (isobar with 2-methylpalmitate)	1.13	0.2341
1,3-dipalmitoylglycerol	0.92	0.6146	citrulline	0.91	0.2341
glycerol 2-phosphate	1.05	0.6147	1-linoleoylglycerophosphoinositol*	0.88	0.2360
cis-vaccenate (18:1n7)	1.03	0.6170	1-stearoylglycerophosphoinositol	0.85	0.2362
ophthalmate	0.95	0.6199	2-stearoylglycerophosphoinositol*	0.77	0.2379
C-glycosyltryptophan*	0.98	0.6295	phenylalanylaspartate	1.20	0.2430
palmitoyl sphingomyelin	0.97	0.6322	phenylalanylglycine	1.17	0.2453
4-hydroxybutyrate (GHB)	1.21	0.6342	flavin adenine dinucleotide (FAD)	0.94	0.2460
dihomo-linolenate (20:3n3 or n6)	1.02	0.6374	guanosine	1.15	0.2508
gamma-glutamylglutamine	1.02	0.6444	glucose-6-phosphate (G6P)	0.75	0.2558
tryptophan	0.98	0.6517	1-palmitoylglycerophosphoethanolamine	0.88	0.2640
anserine	0.97	0.6551	arachidate (20:0)	1.35	0.2810
spermidine	0.92	0.6560	palmitate (16:0)	1.12	0.2851

cysteine	0.96	0.6658	myristoleate (14:1n5)	1.28	0.2854
5-hydroxyindoleacetate	1.04	0.6677	glucuronate	0.86	0.2868
lysine	1.02	0.6689	campesterol	0.87	0.2890
adenosine	0.96	0.6692	coenzyme A	0.90	0.2938
stearoyl sphingomyelin	0.97	0.6758	phenol sulfate	0.76	0.2962
1-stearoylglycerophosphocholine	0.78	0.6793	3-indoxyl sulfate	0.85	0.2980
hydroxybutyrylcarnitine*	0.96	0.6919	enterolactone	0.81	0.2987
isovalerylcarnitine	0.95	0.6997	glycine	0.95	0.2994
aspartate	0.98	0.7067	hypoxanthine	0.93	0.3012
cytidine 5'-diphosphocholine	0.99	0.7082	tryptophan	0.95	0.3094
ascorbate (Vitamin C)	1.03	0.7107	propionylcarnitine	1.11	0.3100
succinylcarnitine	1.05	0.7190	hippurate	0.84	0.3172
carnosine	1.04	0.7191	2-phosphoglycerate	0.85	0.3173
arachidonate (20:4n6)	1.01	0.7226	beta-alanine	1.14	0.3176
ribose	0.96	0.7293	pantothenate	0.91	0.3179
sarcosine (N-Methylglycine)	1.03	0.7409	pipecolate	0.89	0.3193
docosapentaenoate (n6 DPA; 22:5n6)	0.98	0.7478	glucose	0.95	0.3196
5-methylthioadenosine (MTA)	1.01	0.7483	N-acetylglucosamine 6-phosphate	0.92	0.3209
1-palmitoylglycerol (1-monopalmitin)	0.98	0.7510	glycylleucine	1.12	0.3211
Isobar: fructose 1,6-diphosphate, glucose 1,6-diphosphate	1.04	0.7592	1,3-dihydroxyacetone	0.90	0.3218
sphingosine	0.96	0.7632	adenosine 2'-monophosphate (2'-AMP)	1.07	0.3246
3-indoxyl sulfate	0.95	0.7757	myristate (14:0)	1.18	0.3282
mead acid (20:3n9)	1.02	0.7761	1-palmitoylglycerophosphoinositol*	0.89	0.3285
pantothenate	0.99	0.7813	ribose	0.91	0.3346
allo-threonine	1.05	0.7885	1-stearoylglycerophosphoethanolamine	0.90	0.3403

mannitol	1.04	0.7938	beta-muricholate	1.19	0.3446
ethanolamine	0.96	0.8018	cysteine-glutathione disulfide	1.24	0.3471
linolenate [alpha or gamma; (18:3n3 or 6)]	1.03	0.8036	cysteinylglycine	0.91	0.3482
7-beta-hydroxycholesterol	0.97	0.8090	mead acid (20:3n9)	1.19	0.3512
histidine	0.99	0.8137	spermidine	1.06	0.3514
3-phosphoserine	0.97	0.8239	pseudouridine	0.92	0.3522
hypoxanthine	0.99	0.8298	glutathione, reduced (GSH)	0.94	0.3532
N-acetylglutamate	0.99	0.8369	gamma-glutamylphenylalanine	1.11	0.3553
glycerophosphorylcholine (GPC)	1.02	0.8436	creatine	0.83	0.3573
adrenate (22:4n6)	0.96	0.8451	2-hydroxystearate	0.86	0.3578
leucine	0.99	0.8513	glycerol 3-phosphate (G3P)	1.13	0.3595
erythronate*	1.02	0.8572	isoleucylglycine	1.07	0.3632
linoleate (18:2n6)	0.98	0.8587	1-pentadecanoylglycerophosphocholine*	0.87	0.3651
glutamine	1.01	0.8591	1-oleoylglycerophosphoethanolamine	0.92	0.3716
inosine	1.01	0.8653	2-hydroxybutyrate (AHB)	0.89	0.3817
arginine	0.99	0.8730	2-palmitoylglycerophosphoethanolamine*	0.87	0.3821
N-acetylglutamine	0.99	0.8770	equol sulfate	0.90	0.3854
eicosenoate (20:1n9 or 11)	1.02	0.8771	pentadecanoate (15:0)	0.88	0.3929
laurate (12:0)	0.99	0.8782	2-oleoylglycerophosphoethanolamine*	0.87	0.4008
phosphopantetheine	0.98	0.8822	allantoin	1.08	0.4014
palmitoleate (16:1n7)	1.01	0.8904	inosine 5'-monophosphate (IMP)	0.69	0.4110
gamma-glutamylglutamate	0.99	0.8918	S-methylglutathione	1.07	0.4117
1-oleoylglycerophosphoinositol*	0.99	0.9006	threonate	1.19	0.4123
2-oleoylglycerophosphoserine*	1.01	0.9077	valylglycine	1.18	0.4172
phosphoethanolamine	1.00	0.9223	adenosine 3',5'-diphosphate	0.91	0.4211

2-hydroxyglutarate	1.00	0.9329	Isobar: betaine aldehyde, N-methyldiethanolamine	0.89	0.4224
hydroxyisovaleroyl carnitine	1.00	0.9440	dihydroxyacetone phosphate (DHAP)	0.90	0.4284
alpha-hydroxyisovalerate	0.99	0.9495	ergothioneine	1.04	0.4332
10-nonadecenoate (19:1n9)	0.99	0.9508	nicotinamide adenine dinucleotide (NAD+)	0.95	0.4364
5-oxoproline	1.00	0.9517	glutarylcarnitine (C5)	1.06	0.4386
2-aminoadipate	1.01	0.9549	genistein	0.81	0.4485
phosphate	1.00	0.9550	1-oleoylglycerophosphoinositol*	1.07	0.4538
dehydroascorbate	1.01	0.9588	malate	1.07	0.4557
beta-alanine	1.00	0.9628	5-oxoproline	1.05	0.4609
arabinose	1.00	0.9693	3-(4-hydroxyphenyl)lactate	0.91	0.4610
isobutyrylcarnitine	1.00	0.9861	13-HODE + 9-HODE	1.11	0.4616
flavin adenine dinucleotide (FAD)	1.00	0.9877	adenosine 5'-monophosphate (AMP)	1.13	0.4622
taurine	1.00	0.9889	pyridoxate	0.94	0.4642
1-docosahexaenoylglycerol (1-monodocosahexaenoin)	1.00	0.9915	gamma-glutamyltyrosine	0.90	0.4654
12-HETE	1.00	0.9917	aspartylleucine	1.24	0.4663
1-oleoylglycerol (1-monoolein)	1.00	0.9955	glycylproline	1.05	0.4721
5-aminovalerate	1.00	0.9961	stearate (18:0)	1.06	0.4740
			2-arachidonoyl glycerol	0.92	0.4797
			succinylcarnitine	1.10	0.5009
			gamma-aminobutyrate (GABA)	0.89	0.5060
			1-docosahexaenoylglycerol (1-monodocosahexaenoin)	1.07	0.5062
			gamma-glutamylvaline	0.92	0.5087
			S-lactoylglutathione	0.91	0.5139
			ribose 5-phosphate	1.07	0.5209
			caproate (6:0)	0.92	0.5251
			phenylalanylserine	1.06	0.5319

arginine	1.16	0.5344
phenylpropionylglycine	1.11	0.5345
cytidine 5'-monophosphate (5'-CMP)	0.96	0.5444
N-acetylglucosamine	1.09	0.5490
valine	1.03	0.5553
carnitine	1.02	0.5562
2'-deoxyinosine	1.07	0.5567
thiamin (Vitamin B1)	1.04	0.5603
kynurenate	0.93	0.5738
glutarate (pentanedioate)	0.88	0.5755
phenylacetylglycine	0.89	0.5843
caprylate (8:0)	1.06	0.5995
maltohexaose	1.04	0.6081
alanylvaline	1.04	0.6104
alanylleucine	1.05	0.6122
beta-hydroxypyruvate	1.05	0.6174
3-phosphoglycerate	1.05	0.6210
glutamate	1.05	0.6230
butyrylglycine	0.92	0.6315
C-glycosyltryptophan*	0.97	0.6319
methionine	0.97	0.6332
maltotetraose	1.04	0.6349
ethanolamine	0.93	0.6368
Isobar: fructose 1,6-diphosphate, glucose 1,6-diphosphate	0.94	0.6413
4-methyl-2-oxopentanoate	0.95	0.6614
biopterin	1.04	0.6622
1-arachidonoylglycerophosphoethanolamine*	0.96	0.6648
1-arachidonylglycerol	1.04	0.6663

dihydrocholesterol	0.94	0.6705
1-stearoylglycerophosphoglycerol	1.09	0.6715
7,8-dihydrofolate	0.98	0.6752
1-stearoylglycerol (1-monostearin)	1.06	0.6860
adenylosuccinate	1.11	0.6867
glutamine	1.03	0.6941
S-adenosylmethionine (SAM)	1.04	0.6951
isoleucine	0.98	0.6960
1-palmitoleoylglycerophosphoethanolamine*	0.94	0.6974
alanylphenylalanine	1.04	0.6984
hydroxyisovaleroyl carnitine	0.97	0.6993
adenosine 5'diphosphoribose	1.06	0.7178
nicotinamide	1.02	0.7213
hypotaurine	0.93	0.7227
phosphate	1.01	0.7240
leucylglycine	1.04	0.7302
uridine monophosphate (5' or 3')	1.05	0.7374
aspartate	1.02	0.7383
tyrosine	1.02	0.7442
2-arachidonoylglycerophosphoinositol*	0.96	0.7507
threonine	1.02	0.7551
stachydrine	1.04	0.7551
glycerophosphorylcholine (GPC)	0.97	0.7645
imidazole propionate	1.07	0.7719
guanosine 5'-monophosphate (5'-GMP)	1.04	0.7741
sorbitol	1.04	0.7790
glycerol 2-phosphate	1.03	0.7979

betaine	0.98	0.8013
serine	0.99	0.8102
2-methylbutyrylcarnitine (C5)	0.97	0.8150
cytidine	1.02	0.8206
alpha-tocopherol	1.04	0.8215
3-methylglutarylcarnitine (C6)	0.96	0.8250
1-methylimidazoleacetate	0.96	0.8325
cholesterol	1.01	0.8336
dihydrobiopterin	1.01	0.8381
3-dehydrocarnitine*	0.99	0.8486
phenylalanine	1.00	0.8775
riboflavin (Vitamin B2)	0.99	0.8876
alanine	0.99	0.8942
glutathione, oxidized (GSSG)	0.99	0.8955
ascorbate (Vitamin C)	1.02	0.8971
1-arachidonoylglycerophosphoinositol*	0.99	0.9000
N-glycolylneuraminate	1.02	0.9037
maltopentaose	1.00	0.9058
phosphoethanolamine	0.99	0.9061
heme	1.05	0.9090
glycocholate	0.97	0.9105
nicotinate	0.99	0.9141
5-methylthioadenosine (MTA)	1.01	0.9163
xylonate	0.98	0.9185
tigloylglycine	0.98	0.9222
isobutyrylcarnitine	0.98	0.9282
lactate	1.00	0.9435
3-hydroxydecanoate	1.01	0.9445

hexanoylglycine	1.01	0.9474
dimethylarginine (SDMA + ADMA)	1.00	0.9479
UDP-galactose	1.01	0.9536
acetylcarnitine	0.99	0.9583
fumarate	1.00	0.9651
2-aminoadipate	0.99	0.9690
gluconate	1.01	0.9695
isoleucylglutamine	1.01	0.9703
ornithine	1.00	0.9717
Isobar: ribulose 5-phosphate, xylulose 5-phosphate	1.00	0.9747
3'-dephosphocoenzyme A	1.00	0.9804
glycyltyrosine	1.00	0.9882
2-linoleoylglycerol (2-monolinolein)	1.00	0.9885

Annex I

Clinical, polysomnographic and genome-wide association analyses of narcolepsy with cataplexy: a European Narcolepsy Network study

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Summary

The aim of this study was to describe the clinical and PSG characteristics of narcolepsy with cataplexy and their genetic predisposition by using the retrospective patient database of the European Narcolepsy Network (EU-NN). We have analysed retrospective data of 1099 patients with narcolepsy diagnosed according to International Classification of Sleep Disorders-2. Demographic and clinical characteristics, polysomnography and multiple sleep

latency test data, hypocretin-1 levels, and genome-wide genotypes were available. We found a significantly lower age at sleepiness onset (men versus women: 23.74 ± 12.43 versus 21.49 ± 11.83, $P = 0.003$) and longer diagnostic delay in women (men versus women: 13.82 ± 13.79 versus 15.62 ± 14.94, $P = 0.044$). The mean diagnostic delay was 14.63 ± 14.31 years, and longer delay was associated with higher body mass index. The best predictors of short diagnostic delay were young age at diagnosis, cataplexy as the first symptom and higher frequency of cataplexy attacks. The mean multiple sleep latency negatively correlated with Epworth Sleepiness Scale (ESS) and with the number of sleep-onset rapid eye movement periods (SOREMPs), but none of the polysomnographic variables was associated with subjective or objective measures of sleepiness. Variant rs2859998 in UBXN2B gene showed a strong association ($P = 1.28E-07$) with the age at onset of excessive daytime sleepiness, and rs12425451 near the transcription factor TEAD4 ($P = 1.97E-07$) with the age at onset of cataplexy. Altogether, our results indicate that the diagnostic delay remains extremely long, age and gender substantially affect symptoms, and that a genetic predisposition affects the age at onset of symptoms.

Keywords: age at onset; diagnostic delay; gender; genome-wide association

Introduction

Narcolepsy with cataplexy (NC) affects ~20 per 100 000 individuals, with an incidence of ~0.3–0.6 per 100 000 person-years (Longstreth *et al*., 2007; Partinen and Hublin, 2011; Poli *et al*., 2013). Evidence indicates that NC is caused by the loss of ~70 000 hypocretin cells in the hypothalamus, and the best biological marker for human narcolepsy is the reduction (or complete deficiency) of hypocretin-1 in the cerebrospinal fluid (CSF; Nishino *et al*., 2000).

NC displays a strong genetic predisposition: more than 92% of Caucasian patients (Mignot *et al.*, 2001) carry the human leukocytes antigen (HLA)–*DQB1*06:02*, while HLA–*DQB1*06:03* allele confers a strong protection (Hor *et al.*, 2010). The tight association with specific HLA alleles, the recent discovery of circulating anti-TRIBBLES-2 antibodies (Cvetkovic-Lopes *et al.*, 2010), the potentially elevated antistreptolysin O titers close to disease onset (Aran *et al.*, 2009), the association with a polymorphism of T-cell receptor alpha gene and a polymorphism of the purinergic receptor *P2RY11* gene (Kornum *et al.*, 2011) are strong indications of an autoimmune origin. However, the exact mechanism of such an autoimmune process remains unknown.

Despite major advances in understanding the pathophysiology of the disease and despite the fact that narcolepsy is a serious chronic sleep disorder with a major impact on performance, social relations, quality of life and socio-economic burden (Jennum *et al.*, 2012), many cases remain undiagnosed (Dodel *et al.*, 2007; Ozaki *et al.*, 2008; Vignatelli *et al.*, 2004). Variation in severity of the condition with incomplete or atypical forms and variable symptoms might contribute to under-diagnosis. Also, narcolepsy may be misdiagnosed as another sleep disorder, psychiatric disorder, epilepsy or side-effect of medication, or by lack of knowledge of the clinical characteristics (Morrish *et al.*, 2004). In addition, the exact nosology of narcolepsy is still controversial for different reasons.

1. Narcolepsy with cataplexy can be diagnosed by history alone (American Academy of Sleep Medicine, 2005) but this approach may not be accurate (Morrison *et al.*, 2011), as excessive daytime sleepiness (EDS) is a common feature of many sleep disorders, and cataplexy has common characteristics with experiences reported by healthy subjects (Anic-Labat *et al.*, 1999; Overeem *et al.*, 2011). Even if EDS in narcolepsy is

different from that in other sleep disorders, a precise description is missing in the international criteria for NC. Nevertheless, significant differences exist between the cataplexy-like episodes and clear-cut cataplexy, such as the triggers and the muscles involved (Anic-Labat *et al*., 1999; Sturzenegger and Bassetti, 2004). Additional symptoms, such as hypnagogic or hypnopompic hallucinations (HH) and sleep paralysis (SP) are frequent in the general population (Ohayon *et al*., 1996, 1999; Sharpless and Barber, 2011).

2. Patients with NC may test negative in the proposed multiple sleep latency test (MSLT) requirements. On the other hand, patients with sleep apnea (Chervin and Aldrich, 2000) and even normal subjects (Mignot *et al*., 2006) may show short MSLT latencies and sleep-onset rapid eye movement periods (SOREMPs). Even if the prevalence of SOREMPs during MSLT in the general population has not been exhaustively studied, some evidence (Singh *et al*., 2006) suggests that 3.9% of the general population can be positive for multiple SOREMPs. Aldrich underlined that 30% of patients in an average sleep laboratory fulfilling both criteria, a mean sleep latency <5 min and two or more SOREMPs, do not have narcolepsy (Aldrich *et al*., 1997). With the more recent limit of <8 min this percentage will even be greater.
3. From a clinical point of view, the spectrum of NC is still far from being clearly delineated. The nosological limits between narcolepsy without cataplexy (presenting with all narcolepsy symptoms but cataplexy), with similar polysomnographic (PSG) and MSLT abnormalities (Dauvilliers *et al*., 2003; Mignot *et al*., 2002), idiopathic hypersomnia without long sleep time, and non-organic hypersomnia (Kaplan and Harvey, 2009) are in some cases imprecise. There are many common clinical aspects

between narcolepsy without cataplexy and idiopathic hypersomnia without long sleep time (note also that the delay between EDS and cataplexy onset may be highly variable), which may point to the need of confirming proposed diagnostic criteria (Bassetti and Aldrich, 1997; Bassetti *et al*., 2003; Billiard, 2007).

4. Although NC is tightly associated with HLA, genotype can only support diagnosis, as the main susceptibility allele (*DQB1*06:02*) is also found in about 25% of healthy controls (Mignot *et al*., 2001).
5. Although NC is associated with low or undetectable levels of hypocretin-1 in the CSF (normal hypocretin levels in NC are exceptional; Knudsen *et al*., 2010), this measure is not routinely used in clinical practice. In addition, low CSF hypocretin-1 levels were described in other neurological or sleep disorders, such as in certain cases of Guillain–Barré syndrome, anti-Ma encephalitis, late stage of Parkinson's disease, head trauma and in Prader–Willi syndrome (Baumann *et al*., 2008; Fronczek *et al*., 2009; Mignot *et al*., 2002; Overeem *et al*., 2004; Ripley *et al*., 2001b).
6. Many studies emphasize the increased body mass index (BMI) in patients with narcolepsy, as compared with either idiopathic hypersomnia or the general population (Kok *et al*., 2003; Poli *et al*., 2009), but the relationship between the metabolic alterations and the duration of untreated disease was not explored.

Given the preceding limitations, the aim of the present study was to provide a reference detailed clinical and PSG description of a large sample of patients with NC and their eventual genetic predisposition. A major aim was to establish the relationship between symptoms and the diagnostic delay. To the best of our knowledge, our sample is the largest population of well-defined NC ever reported.

Patients and Methods

Patients were diagnosed in affiliated sleep centers of the European Narcolepsy Network (EU-NN): France (n = 339); the Netherlands (n = 219); Germany (n = 185); Spain (n = 157); Italy (n = 68); Switzerland (n = 52); Denmark (n = 41); Poland (n = 30); and Slovakia (n = 8), with a final sample of 1099 patients. The data were gathered as a retrospective EU-NN database during a European genome-wide association study (GWAS; Hor *et al.*, 2010) comparing sporadic cases of narcolepsy with HLA-matched controls. Patients were examined by physicians experienced with narcolepsy, and the diagnosis was based on diagnostic criteria of the ICSD-2 (American Academy of Sleep Medicine, 2005; Billiard, 2007). All patients had narcolepsy with unambiguous clear-cut cataplexy, defined as sudden episodes of muscle weakness triggered by emotions. Given the differences between sporadic and familial cases (e.g. the frequency of HLA–*DQB1*06:02* negative and CSF hypocretin-1 normal cases; Mignot, 1998; Mignot *et al.*, 2002), suggesting a potential different pathogenesis (as demonstrated in the canine model of narcolepsy; Ripley *et al.*, 2001a), only sporadic cases were included. All patients were from European origin and were HLA–*DQB1*06:02* positive. The data were derived from a number of research projects, some published (e.g. Dauvilliers *et al.*, 2001; Hor *et al.*, 2010). Local ethics committees approved the recruitment of patients for research protocols, and all patients gave their consent to participate.

Patients were investigated in terms of the following.

1. Demographic characteristics: date of birth, gender, height, weight, BMI at diagnosis. Circumstances at onset (triggering factors) were not available in most patients.
2. Age at EDS onset and age at cataplexy onset. We defined the age at onset of NC as the age at occurrence of EDS and/or cataplexy, determined during the clinical interview.

3. Frequency of cataplexy attacks at diagnosis. The frequency of cataplexy was assessed by a scale from 1 to 5, reporting rare to very frequent cataplexy attacks (Dauvilliers *et al*., 2001): 1 = one or less cataplexy attacks per year; 2 = more than one cataplexy attack per year but less than one per month; 3 = more than one attack per month but less than one per week; 4 = more than one per week but less than one per day; 5 = at least one cataplexy attack per day.
4. Epworth Sleepiness Scale (ESS) score at diagnosis.
5. Polysomnographic variables [including apnea–hypopnea index (AHI) and periodic leg movements during sleep index (PLMSI) when available] and MSLT results (mean sleep latency, number of SOREMPs) at diagnosis. Even if the recording procedures were different amongst centers, most of the patients underwent nocturnal in-lab PSG followed by an MSLT as part of the diagnostic evaluation. For PSG and MSLT, sleep latency was defined as the time from lights off to the first epoch scored as sleep. A SOREMP was defined as the occurrence of an epoch of REM sleep within 15 min after the first epoch scored as sleep. Although MSLT was performed according to standard methods (Carskadon *et al*., 1986), the number of scheduled naps could be variable. To standardize the results, we calculated the percentage of SOREMPs of the total number of naps: percentage of naps with SOREMPs = [(number of SOREMPs/number of MSLT sessions) × 100].
6. Cerebrospinal fluid hypocretin-1 (measured by Phoenix RIA kit) level when available.
7. Human leukocytes antigen–*DQB1* genotyping was available from affiliated HLA or blood centers (at least 4 digits typing by standard techniques).

8. Associated features, with particular attention to symptoms frequently reported with narcolepsy: SP; HH; and poor nocturnal sleep.
9. Co-morbidities (sleep-related, somatic or psychiatric) and treatment when available.
10. Genome-wide association study: to test genetic associations with clinical traits; age and gender were used as covariates whenever they showed nominally significant association with the tested trait ($P < 0.05$). Continuous clinical phenotypes were inverse normal quantile transformed before applying linear regression [including relevant covariates and single nucleotide polymorphism (SNP) imputed allele dosage]. For dichotomous clinical traits logistic regression was used.

Statistical analysis

Descriptive analyses were performed for: gender; age at diagnosis; age of symptom onset; symptoms at diagnosis; BMI, ESS, PSG and MSLT results; hypocretin-1 level; frequency of co-morbidities and symptomatic treatments. The interval between symptoms' onset and diagnosis was calculated. The gender effect on demographic (age at symptoms' onset, age at diagnosis, diagnostic delay, BMI) and clinical variables (PSG, MSLT, sleepiness, frequency of cataplexy and hypocretin level) was analysed using *t*-test/Kruskal–Wallis/χ^2 test. Additional statistical analyses were carried out using, when appropriate, *t*-test/anova/Kruskal–Wallis to test the effect of origin, BMI, sleepiness and frequency of cataplexy. A possible interaction between BMI and gender was evaluated by two-way anova. Because not all variables were available in all patients, the number of patients in each test was different. To verify that the outcome of our statistical tests was not affected by sub-sampling for each variable, the effects of age and gender on BMI, diagnostic delay

and sleep characteristics were analysed by multivariate regression analysis in a reduced sample with complete information for variables of interest ($n = 611$). This analysis did not reveal any major difference as compared with analyses including the maximum number of patients for each variable. Correlation coefficient (Pearson or Spearman) was used to assess the relationship between demographic and clinical variables. Statistical significance for all tests was assumed when $P < 0.05$. For the entire sample and separately, for each gender, we have performed principal component analysis taking into account demographic, clinical, PSG, MSLT and laboratory data. All statistical tests were computed using PASW 18.0 Statistics (IBM SPSS Statistics, Armonk, NY, USA).

Results

Demographic data

Data from 1099 patients were analysed. Table 1 shows demographic characteristics of our sample. The changes in sample size occurred because of missing data. Discrepancies or absence of information in the charts were labeled as data missing. For some data, manifestly outlier values were removed [e.g. total sleep time (TST) <2 h, MSLT latency >20 min, etc.].

In our sample, 54.8% of the patients were men and 45.2% women. The birth distribution ranged from 1905 to 2004. No predominance of any month or season was observed for birth. The mean age at diagnosis was 36.9 ± 17.1 years (range: 4–87 years). The age of diagnosis varied depending on the country of origin, with a mean age at diagnosis ranging from 33.1 ± 16.8 years in France to 43.9 ± 17.6 years in Spain (one-way anova for factor 'origin', $F = 9.234$, $P < 0.001$), but it was similar in women (36.9 ± 16.9 years) and men (36.8 ± 17.4 years). Age at diagnosis and diagnostic delay were strongly correlated ($r = 0.799$,

$P < 0.001$). The strength of this correlation was maintained even after excluding patients younger than 20 years old from the analysis ($r = 0.714$, $P < 0.001$).

In most of the patients both EDS and cataplexy appeared at the same time (48.8%), EDS preceded the onset of cataplexy in 43.8% and cataplexy was the first reported symptom in 7.4% of patients only. There were no significant differences between countries or by gender.

Based on clinical interview, the age at EDS onset could be established in 990 patients. The mean age at EDS onset was 22.7 ± 11.9 years (range: 3–80 years; median: 20 years), with a significant gender difference (*t*-test; Mann–Whitney $U = 107\,302$, $P = 0.003$; 23.7 ± 12.4 years in men versus 21.5 ± 11.8 years in women) but no difference between countries. The mean age at cataplexy onset ($n = 685$) was 25.8 ± 12.8 years (range: 5–80 years; median: 24 years), with a normal distribution in both genders. The age of cataplexy onset was significantly different between countries ($F = 3.088$, $P = 0.009$; minimum in Italy, 23.0 ± 12.1 years; maximum in Germany, 32.9 ± 11.3 years). In seven patients, age at symptoms' onset was older than 60 years (EDS was the first symptom or appeared at the same time as cataplexy). The mean delay between EDS onset and cataplexy onset ($n = 678$) was 2.8 ± 8.0 years. The maximum delay observed between EDS and cataplexy onset was 48 years, and the maximum delay between cataplexy onset and EDS was 40 years.

Available data from 738 patients revealed that the mean delay between the onset of the first clinical symptom (EDS or cataplexy) and diagnosis was 14.6 ± 14.3 years (median 10.5 years; range from <1 to 67 years). The delay between disease onset and diagnosis was larger for patients with later age at onset, and for those with EDS as first symptom ($r = 0.169$, $P < 0.001$). An important variability between countries was observed for diagnostic delay ($F = 8.042$, $P < 0.001$), with the lowest delay in France (11.9 ±13.7 years) and the highest one

in Spain (21.1 ± 14.7 years). Nevertheless, diagnostic delay was influenced by the time of diagnosis (for the analysis of the time of diagnosis, groups were created by 10-year intervals). For all countries, cases diagnosed before 1991 have a significantly decreased delay (mean diagnostic delay before 1991 was 8.0 ± 4.7 years, compared with the ones diagnosed after). One explanation could be the accumulation in time of undiagnosed cases. The higher number of cases diagnosed before 1991 was in France, while in Spain the increase in number of cases was observed starting with 1991. For the entire sample, the best predictors of diagnostic delay (forward stepwise regression) were age at diagnosis, first symptom and the frequency of cataplexy (Table 2).

Clinical and laboratory data

BMI

The mean BMI at diagnosis (n = 903) was 27.3 ±5.6 kg m^{-2}. About two-thirds of the patients were overweight or obese (36.5% had a BMI between 25 and 29.9 kg m^{-2}, and 27.2% a BMI > 30 kg m^{-2}). In patients with a normal weight range (BMI < 25 kg m^{-2}) there was a predominance of women (men versus women: 41.8% versus 58.2%, $\chi^2 = 40.867$, $P < 0.001$), for overweight patients a predominance of men was observed (BMI between 25 and 29.9 kg m^{-2}, men versus women: 66.7% versus 33.3%; BMI > 30 kg m^{-2}, men versus women: 54.5% versus 45.5%; $\chi^2 = 21.023$, $P < 0.001$). Patients with a normal BMI had a lower age at diagnosis, age of first symptom onset and diagnostic delay. To control for gender by BMI interaction, the age at diagnosis, age at symptom onset and diagnostic delay were also analysed by two-way anova. Results indicated that gender and BMI affected all three variables, but none was affected by gender by BMI interaction ($P > 0.3$ for all comparisons).

We also observed significant differences in PSG data [TST, sleep efficiency (SE), slow-wave sleep (SWS) and REM sleep duration are reduced, and S1 duration is increased in overweight and obese patients most probably due to an increase in sleep apnea severity; Table 3]. Nevertheless, none of the variables that assess the severity of sleepiness (the mean sleep latency during MSLT, number of SOREMPs, ESS), or frequency of cataplexy and hypocretin-1 levels, was different between patients with normal BMI versus overweight/obese.

Subjective sleepiness

Subjective sleepiness was assessed using the ESS in 803 patients at the time of diagnosis. The mean ESS was 17.4 ± 3.9 with a median score at 18. Only 38 patients (4.7%) had an ESS in the 'normal ranges' (<11). None of the 'non-sleepy' patients was under stimulant medication at diagnosis. The mean age at diagnosis in this group was not different from that of the group with ESS score > 10 (31.6 ± 14.9 years versus 37.3 ± 17.4 years), but the diagnostic delay was shorter (7.8 ± 7.0 years versus 14.7 ± 14.6 years, $P = 0.011$; Table 4). The first symptom in 'non-sleepy' patients was cataplexy in 12%, higher than in those who declared themselves sleepy (7.2%), but the difference was not statistically significant. The variables correlated with sleepiness were different between these two groups of patients, but the severity of narcolepsy symptoms was similar. The number of SOREMPs was significantly higher in patients with high ESS scores (67.94% in high ESS versus 17.32% in normal ESS, $t = 15.33$, $P < 0.0001$).

Cataplexy

The frequency of cataplexy was assessed on a scale from 1 to 5, from rare to very frequent cataplexy attacks. In our case series ($n = 829$), almost two-thirds of the patients had frequent

or very frequent cataplexy attacks (scores 4 + 5: 62.6%; score 5: 41.9%), and 48 patients (5.8%) had very rare cataplexy attacks. The mean frequency of cataplexy score was 3.7 ± 1.3, which corresponds to more than one attack per month. Patients with severe cataplexy (score 5) had a reduced diagnostic delay (11.9 ± 12.8 years versus 18.3 ± 15.8 years, $P < 0.001$), and the percentage of those who experienced sleepiness, HH and SP was higher in patients with severe cataplexy (Table 5).

Associated features

Hypnagogic or hypnopompic hallucinations were experienced by 63.1% of patients (370 patients), and 52.6% (257 patients) experienced SP; both symptoms were present in 43.6% (175 patients). Only 28.2% (303 patients) of the entire patient population reported either SP or HHs alone (Table 6).

PSG features

Table 7 shows PSG and MSLT characteristics of patients with NC. The mean TST was 411.7 ± 80.4 min, with a mean SE of 83.8 ± 11.5%; 42.8% had abnormal SE < 85%. The age at diagnosis was negatively correlated with TST ($r = -0.389$, $P < 0.001$), SE ($r = -0.335$, $P < 0.001$), SWS duration ($r = -0.293$, $P < 0.001$) and REM sleep duration ($r = -0.209$, $P < 0.001$), and positively with S1 duration ($r = 0.315$, $P < 0.001$). The mean number of stage shifts was 108.3 ± 64.6, and the mean wake after sleep onset was 59.5 ± 43 min. The mean sleep-onset latency (SOL) was 10.3 ± 23.8 min, with a mean REM sleep latency (REM SL) of 54.6 ± 65.30 min. Thirty-five percent of patients had a SOREMP during the night PSG (REM SL < 15 min), and only 14.2% had a REM SL between 90 and 150 min, with some patients showing prolonged REM latency (REM SL > 150 min; 9.1%). The distribution of REM SL

was bimodal, with a first peak between 0 and 10 min after sleep onset, and a second less marked peak at 50–70 min. Women had a better SE, longer S2 and SWS duration, and shorter S1.

To evaluate whether the ESS and/or MSLT results are influenced by night sleep, a multiple regression analysis was performed, controlling for age at diagnosis and gender. None of the variables was retained in the analysis, suggesting that changes in sleep structure or duration are due to age and gender, but not to the condition.

The mean sleep latency during MSLT in 927 patients (without treatment) was 3.9 ± 3.0 min, with a median at 3 min, indicating severe sleepiness; 92% of the patients had a sleep latency < 8 min (Table 7). Although small, the mean sleep latency at MSLT was significantly shorter in women (3.68 ± 2.7 min versus 4.10 ± 3.24 min in men). The mean percentage of SOREMPs during the MSLT was 65.9 ± 27.2%, which corresponds roughly to 3 SOREMPs during a 5-naps MSLT. However, 3.9% of the sample had no SOREMPs during the MSLT, and an additional 5.7% had 20% SOREMPs, which corresponds to 1 SOREMP during a 5-naps MSLT. Laboratory criteria currently used to diagnose narcolepsy (sleep latency shorter than 8 min and at least 2 SOREMPs) were present in 90.3% of patients. We found a negative correlation between the mean sleep latency at the MSLT and the number of SOREMPs ($r = -0.326$, $P < 0.001$). Reduced MSLT mean sleep latency predicted SOREMPs ($r = -0.326$, $P < 0.001$). The MSLT latency was negatively correlated with the ESS ($r = -0.306$, $P < 0.001$).

CSF hypocretin-1

Cerebrospinal fluid hypocretin-1 measurements were available in 294 patients (Table 7). In 96.3% of the patients hypocretin-1 levels were undetectable (<40 pg mL^{-1}, $n = 252$) or

<110 pg mL^{-1} ($n = 31$). In the remaining 11 patients, intermediate (four cases) to normal levels (seven cases) were obtained (from 118 to 400 pg mL^{-1}). There were no significant correlations between hypocretin-1 levels (analysed as bivariate parameter: <40 pg/ml and more than 40 pg/ml) and variables of interest; there were no differences in clinical features between patients with undetectable and those with hypocretin-1 levels between 40 and 110 pg mL^{-1}. Regarding the 11 patients with intermediate to normal values of hypocretin-1, the only significant difference was observed in the frequency of cataplexy, which was lower (frequency code 1–2: 42.9% versus 11% in patients with low/undetectable levels of hypocretin-1, $P = 0.004$).

Effect of age and gender on NC features and diagnostic delay

Diagnosis is more delayed in women of all age groups (two-way anova for 'age group' and 'gender', $P < 0.001$; no interaction; men versus women: 13.8 ± 13.8 years versus 15.6 ± 14.9 years), independently from the origin or the time at diagnosis. Stage 1 was increased in men in all age groups (men versus women 16.5 ± 10.9% versus 13.0 ± 9.2%; Mann–Whitney U: 55949, $P < 0.001$). Younger men were sleepier than younger women (ESS in men 20–30 years old versus women 17.4 ± 3.8 versus 16.8 ± 3.9, $P = 0.04$), but with aging women declared themselves sleepier (ESS in men >60 years old versus women 17.6 ± 3.8 versus 18.8 ± 3.8, $P = 0.034$). The same pattern was observed in parameters correlated with sleepiness: mean SOL during MSLT (young men versus young women 3.7 ± 2.7 min versus 4.04 ± 2.6 min, $P = 0.04$; men > 60 years old versus women 4.1 ± 3.6 versus 3.9 ± 2.8, $P = 0.53$) and SOREMPs (young men versus young women 67.3 ± 28.1 versus 63 ± 29; men > 60 years old versus women 59.0 ± 26.2 versus 70.8 ± 25.0, $P = 0.014$).

Principal component analysis

For variables relevant from a clinical point of view we performed a principal component analysis, varimax rotation with Kaiser normalization (KMO measure of sample adequacy 0.611; Bartlett's test of sphericity: chi square 1010.20; $P < 0.001$). This analysis was performed for the entire sample and, separately, for each gender. Table 8 presents the major extracted components (eigenvalue > 1; no restriction on selected number of components). For the entire sample, eight components accounted for 67.05% of phenotype variability. By gender analysis retrieved only seven components for each gender.

For the entire sample, the first major component (% variance explained = 14.33%) included age- and PSG-related variables: age at diagnosis; diagnostic delay and non-REM sleep stage 1 (positively); TST and SE (negatively; Table 8). The second component (% variance explained = 12.68%) accounted for sleepiness, with ESS score, presence of EDS, and number of SOREMPs during MSLT (positively) and mean MSLT (negatively). Components three and four (% variance explained = 7.7% and 7.6%, respectively) were represented by sleep variables. The frequency of cataplexy was extracted (positively) together with the presence of sleepiness, HHs and SP as part of component five (% variance explained = 7.1%). First symptom, diagnostic delay and BMI, SOL, and CSF hypocretin-1 levels with stage 1 (positively) and mean MSLT (negatively) were extracted separately within components 6–8. By gender analysis revealed effects that could not be assessed by other analysis. Compared with the entire sample, the most important variability in men was due to sleepiness and its correlates. In men, diagnostic delay was influenced by the severity of cataplexy and the first symptom (if cataplexy was the first symptom, the diagnostic delay was shorter). In women, those who had cataplexy as the first symptom had a lower REM-onset latency.

Other sleep-related disorders

Respiratory parameters were not systematically assessed in most sleep laboratories when evaluating a patient suspected of NC. In 240 patients detailed breathing parameters were available. The mean AHI was 5.1 ± 10.6 per hour, with 26.3% having an AHI ≤ 5 per hour, 8.7% an AHI between 5 and 15 per hour, and 4.1% an AHI ≥15 per hour. The AHI was higher in men than in women (7.63 ± 13.3 versus 3.3 ± 6.7, $P = 0.007$), and correlated with BMI ($r = 0.208$, $P = 0.002$). No significant correlation was found with sleepiness (MSLT latency: $r = 0.012$, $P = 0.86$; ESS: $r = 0.36$, $P = 0.617$). Patients with AHI > 30 per hour tended to be older than those with AHI <30 per hour (40.5 ± 23 versus 35.3 ± 17.2, $P = 0.22$), and had higher BMI (30.6 ± 5.6 versus 27 ± 5.4, $P = 0.043$).

The PLMSI was available in 174 patients only. The mean PLMSI was 1.5 ± 25.8 per hour of sleep, with a median at 4.6 per hour; 49.4% had a PLMI > 5 per hour and 30.4% a PLMI > 15 per hour.

In a subgroup of 295 patients, the presence of REM sleep behavior disorder (RBD; confirmed during diagnostic PSG or clinically suspected) was established in 46% of men and 54% of women. No differences between patients with NC with and without RBD were found for sex distribution, age at diagnosis, ESS score or mean latency during MSLT.

Two-hundred and seventy patients were evaluated for other parasomnias than RBD. The most frequent parasomnias were: sleep talking (17%); arousal disorders (confusional arousals, sleepwalking, sleep terrors, in 8.1%); and bruxism (5.9%). Four patients (1.4%) exhibited characteristics of sleep-related eating disorders: one associated with arousal disorders, another associated with sleep talking, and two without other associated parasomnias.

Genome-wide association

To verify if any gene variant may be associated with narcolepsy phenotypes, we performed a GWAS in 585 patients who had been genotyped as part of a case–control GWAS recently published (Hor *et al*., 2010). Among potential associations, 13 top hits were genotyped in 387 additional narcolepsy patients for replication. Table 9 shows the associations between the top 13 hits for the original, the replication and the whole sample metanalysed. None of the selected variants reached a genome-wide significance level ($P < 5E-08$). Nevertheless, a SNP (*rs2859998*) within the *UBXN2B*, an adaptor protein required for Golgi and endoplasmic reticulum biogenesis, was strongly associated (metanalysis $P = 1.28E-07$) with the age at onset of EDS. A similar strong association was also found between *rs12425451* near the transcription factor *TEAD4* and the age at onset of cataplexy (metanalysis $P = 1.97E-07$). The later association was the only significant one in the replication sample ($P = 0.048$), suggesting a potential true signal that needs further replication in other independent samples.

Discussion

In this study we were able to analyse phenotypic and genetic data of 1099 sporadic HLA–*DQB1*06:02* positive patients with NC from the retrospective database of the EU-NN connecting sleep laboratories from France, the Netherlands, Germany, Spain, Italy, Denmark, Switzerland, Poland, Slovakia, Czech Republic, Austria, Finland and UK. This is, to our knowledge, the largest published population of well-defined NC. We used this large sample of patients to further describe the clinical picture and the PSG characteristics of NC, to review the major features of NC, and genetic association with all available phenotypes. Below we will discuss the major findings.

Diagnostic phenotypes

Although NC can be clinically diagnosed by the presence of EDS and cataplexy, this is still not accurate enough in many cases, in particular in incomplete and atypical forms, or in patients with co-morbidities such as sleep apnea. In addition, EDS is common in other sleep disorders, and cataplexy, especially incomplete attacks, is not always easy to identify. For these reasons several other phenotypic criteria are widely used to support the diagnosis. Among these, HLA, hypocretin-1 and MSLT criteria are the most widely accepted ones. In our population, all cases had documented cataplexy and were HLA–*DQB1*06:02* positive, allowing us to evaluate the contribution of other phenotypic variables. Although available in only 294 patients (but still the largest sample ever reported), normal hypocretin-1 levels were found in only seven patients (2.38%), indicating, as reported by others, that hypocretin deficiency is the most reliable and sensitive biological marker for NC, even if not practically performed in everyday clinical practice. Among 927 patients with MSLT, 74 (8%) had a mean MSLT > 8 min and 79 (8.5%) had <2 SOREMPs, suggesting that MSLT criteria have a lower diagnostic value. Using the criteria proposed by Poli *et al*. (2013) (EDS and unambiguous cataplexy and MSLT mean sleep latency ≤8 min or MSLT SOREMPs ≥2), 96.9% of the patients met the criteria. Nevertheless, among 79 with < 2 SOREMPs, 41 (53%) had a SOREMP during the preceding night PSG. Adding the PSG SOREMPs to those with 1 SOREMP during MSLT brings the percentage of patients with SOREMPs > 2 up to 94.1%, suggesting a good diagnostic value that can be routinely used, as recently suggested (Andlauer *et al*., 2012). Interestingly, among seven hypocretin-1 normal patients, MSLT data were available in three, and all three had mean MSLT <8 min with more than 2 SOREMPs. The same observation was found in two out of the four patients with intermediate hypocretin-

1 levels. Overall, EDS, cataplexy and *DQB1*06:02* best characterize the condition, in the absence of hypocretin-1 measure. MSLT data together with PSG SOREMPs remain highly valuable, especially if the HLA typing is not available. Also note that in our population mean MSLT negatively correlated with the ESS score ($r = -0.302$, $P < 0.001$). Accordingly, principal component analysis identified ESS, mean MSLT and number of SOREMPs contributing to the same factor with strong correlations between them. In other disorders with EDS as the leading symptom this correlation cannot be demonstrated (Chervin and Aldrich, 1999; Shpirer *et al*., 2006).

Time between narcolepsy onset and diagnosis

The delay between the first symptom and diagnosis has been previously reported to range from the same year to more than 60 years (Morrish *et al*., 2004; Parkes *et al*., 1995), usually more than a decade, with a mean delay between 15 and 17 years after the onset of EDS (Moldofsky *et al*., 2000; Morrish *et al*., 2004). Although it is generally claimed that the diagnostic delay has considerably shortened in recent years, in our population the mean diagnostic delay is still substantial (14.6 ± 14.3 years). Interestingly, patients with older age at onset had longer diagnostic delay, while younger patients (mainly children and adolescents) have the shortest diagnostic delay. Also, patients with EDS as the first symptom have longer diagnostic delay as compared with those with both EDS and cataplexy or cataplexy as the first symptom. Age at symptom onset and age at diagnosis varied amongst countries. Obviously several factors might be involved, including but not limited to differences in the recruitment population (child versus adult patients) and history of narcolepsy research in each country. Another interesting finding is the significant between-country differences in diagnostic delay,

with the shortest in France (11.9 ± 13.7 years) and the longest in Spain (21.1 ± 14.7 years). Nevertheless, the diagnostic delay also depended on the year of diagnosis, with those diagnosed before 1991 having the shortest delay (8.0 ± 4.7 years). This paradoxical finding can be explained by a sharp increase in awareness and the number of narcolepsy specialists in the 1980s. In summary, the best predictors of a short diagnostic delay are the young age at diagnosis, the first symptom including cataplexy, and the higher frequency of cataplexy. These observations are in accordance with previous reports indicating that the year of symptom onset and whether or not cataplexy is one of the initial symptoms show a significant correlation with the diagnosis delay (Morrish *et al*., 2004). Overall, the onset or worsening of cataplexy often prompts patients to look for medical assistance (Rye *et al*., 1998), the interval between symptom onset and diagnosis is greater in patients whose symptom onset was further in the past (Dauvilliers *et al*., 1998), and a reduction in the diagnosis delay is found in patients with a more recent date of birth (Furuta *et al*., 2001).

Age and gender contributions

Gender differences in narcolepsy phenotypes are poorly investigated. Here we found a significantly longer diagnostic delay in women. Also, the age at onset for EDS was lower in women. These differences seem robust as there were no gender differences between countries or the time of diagnosis, while the age at onset for cataplexy was highly significantly different between countries. Mean sleep latency at MSLT was significantly shorter in women, in addition to several PSG and BMI differences. Our findings clearly indicate that narcolepsy symptoms are strongly affected by age and especially gender, needing further investigations.

Multivariate analysis

Using the most typical phenotypes in our multivariate analysis, we identified up to eight factors explaining their variance in a large NC population. As expected, the most significant one included age at diagnosis and diagnostic delay negatively correlated with TST and SE, and positively with the amount of stage 1 non-REM sleep. The second most significant component includes variables related to EDS, with ESS score and number of SOREMPs during MSLT being negatively correlated with mean sleep latency during MSLT. BMI segregated positively with stage 1 non-REM sleep and REM latency at night, and negatively with diagnostic delay and nocturnal REM sleep. Of interest, the levels of hypocretin-1 positively correlated with stage 1 non-REM sleep and negatively with mean latency during MSLT. Note that none of the night PSG variables was associated with subjective (ESS) or objective measures of sleepiness (MSLT), suggesting that EDS in narcolepsy does not result from poor quality night sleep, as already proposed (Broughton *et al.*, 1994). Overall, our analysis successfully identified groups of correlated symptoms, each explaining between 5.8 and 14.33% of phenotypic variance (total variance explained by eight factors = 67%).

Genetics of narcolepsy phenotype

We performed the first GWAS on narcolepsy clinical and laboratory phenotypes. Although many suggestive variants were found, only one, associated with the age at onset of cataplexy, showed a nominal significant association in the replication sample. The major explanation for this negative result is the complexity of the analysed phenotypes where genetic variants explain a small proportion of the phenotypic variance. Also, our original sample (585 patients) is somehow too small to have enough power to detect a genome-wide significant

signal (note also that data for different phenotypes were not available in all 585 patients further reducing the statistical power). Nevertheless, the variant *rs12425451* associated with the age at onset of cataplexy is in the vicinity of the transcription factor TEAD4. TEAD4 is an important transcription factor controlling neuronal fate and survival (Cao *et al*., 2008). This interesting finding needs replication.

Conclusion

In this large sample of well-defined patients with NC, our findings are compatible with previously published studies, extending data collected in smaller case-series. In addition we have identified several unexplored relationships between narcolepsy phenotypes. The long delay between the onset of symptoms and diagnosis implies that many cases still remain undiagnosed, and that major efforts need to be made to spread the knowledge of the disease among the population and in particular among physicians. Note that longer diagnostic delay is found here associated with higher BMI, suggesting that late diagnosis and/or undiagnosed cases are at higher risk for metabolic and cardiovascular diseases. Our findings also emphasize a large heterogeneity in the clinical presentation and laboratory variables of the disease, and point out the fact that accurate diagnosis needs to take into account the full clinical presentation and a critical interpretation of PSG and MSLT results. Although not routinely proposed, the measurement of hypocretin-1 concentration in the CSF could be an important diagnostic tool in dubious cases. Future studies should also take into account the gender as a major contributor.

Finally, we acknowledge several limitations of our study. Although the diagnosis was made in reference sleep laboratories with long-lasting expertise in narcolepsy following virtually identical diagnostic criteria, several procedures vary between laboratories, and complete and

uniform data could not be obtained from all patients included, introducing some diagnostic site effects (a structured prospective database has been launched by the EU-NN in 2010). Some of the parameters analysed in the study (e.g. onset of EDS) were based on the reliance on subjects of correctly recalling symptoms, or in the analysis of retrospective data obtained from medical charts, which may vary according to differences in interpretation or in conducting clinical interviews between sites. We did not compare our patients with a reference population. Comparisons with subjects recruited from the same populations and diagnosed either with other hypersomnias or without any sleep disorder could have allowed identification of the most specific (in terms of specificity and sensitivity) narcolepsy phenotypes and comparisons of co-morbidities or the search for environmental risks factors implicated in the pathophysiology of the disease.

In conclusion, this study provides a detailed description of the clinical and PSG characteristics of a large and homogeneous group of patients with NC. Despite major advances during recent years in our understanding of the neurobiological basis of narcolepsy, NC is still an under-recognized condition. A better knowledge of the nosology of NC may allow earlier diagnosis of this life-long disabling condition.

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Table 1 Demographic characteristics of the sample

Parameter	n *(All)*	*Mean ± SD*	*Men*	*Women*	t/v^2	P
Age at diagnosis (years)	755	36.87 ± 17.13	36.80 ± 17.40	36.91 ± 16.86	NS	NS
Age at EDS onset (years)	990	22.73 ± 11.88	23.74 ± 12.43	21.49 ± 11.84	107 302	0.003*
Age at cataplexy onset (years)	685	25.80 ± 12.84	26.05 ± 13.25	25.45 ± 12.25	NS	
Diagnostic delay	738	14.63 ± 14.31	13.82 ± 13.79	15.62 ± 14.94	60 950	0.044*
First symptom (%)					NS	
Cataplexy	47	7.40	8.10	6.50		
Cataplexy and EDS	310	48.80	51.30	45.50		
EDS	278	43.80	40.60	48.00		
BMI	903	27.34 ± 5.59	27.77 ± 4.56	26.80 ± 6.55	82 629	<0.001
Gender (% men)	590	54.80				

[a]Mann–Whitney *U*, *t*-test for ranks; *t*: *t*-test; χ^2: Fisher's exact test. BMI, body mass index; EDS, excessive daytime sleepiness.

Table 2. Forward stepwise regression for best predictors of diagnostic delay

Step no.	*Vars. entered*	r	R^2	P
1	Age at diagnosis	0.714	0.509	0.006
2	First symptom	0.732	0.536	<0.001
3	Cataplexy frequency	0.738	0.545	<0.001

Table 3. Demographic, clinical and PSG characteristics of the patients, according to BMI

Parameter	n	*BMI < 25 kg m^{-2}*	*BMI 25–29.9 kg m^{-2}*	*BMI > 30 kg m^{-2}*	*F/K–W*	P
Age at diagnosis (years)	699	30.54 ± 15.12	42.30 ± 17.41	39.57 ± 17.04	35.77	<0.001
Age at EDS onset (years)	878	19.94 ± 10.29	25.38 ± 12.85	23.62 ± 12.10	17.66	<0.001
Age at cataplexy onset (years)	642	22.78 ± 11.87	29.52 ± 13.76	25.74 ± 12.30	16.924	<0.001
Diagnostic delay	686	11.02 ± 12.07	16.70 ± 15.40	17.45 ± 15.58	14.547	<0.001
First symptom (%)	597					
Cataplexy		6.70	8.70	6.50	1.003	0.606
Cataplexy and EDS		47.30	46.30	53.50		
EDS		46.00	45.00	40.00		
Gender (% men)		41.80	66.70	54.50	3.536	<0.001
HH (% positive)		65.80	62.50	59.00	2.396	0.302
SP (% positive)		54.80	55.30	50.20	1.501	0.472
HH and SP (% positive)		45.70	45.00	41.30	2.519	0.281
EDS, HH and SP (% positive)		44.40	42.00	38.30	3.041	0.219
ESS score		17.16 ± 3.91	17.63 ± 3.83	17.58 ± 3.91	1.209	0.3
EDS (% positive)		95.10	95.60	95.00	0.094	0.954
Frequency of cataplexy (%)						
1 (one or less cataplexy attacks per year)		4.70	8.90	3.70	2.201	0.333
2 (more than one cataplexy attacks per year, but less than one per month)		13.40	11.70	14.30		
3 (more than one attack per month, but less than one attack per week)		18.10	20.60	16.40		
4 (more than one attack per week, but less than one per day)		22.00	17.50	19.00		
5 (at least one cataplexy attack per day)		41.90	41.20	46.60		
Sleep latency in PSG						
TST	686	427.22 ± 69.70	407.38 ± 82.69	392 ± 80.59	22.969	<0.001
SE	761	86.51 ± 16.99	82.59 ± 11.90	81.76 ± 12.04	35.857	<0.001
Stage1 (% of TST)	722	12.16 ± 8.76	15.26 ± 10.65	16.72 ± 10.72	27.248	<0.001
Stage 2 (% of TST)	721	43.56 ± 11.27	44.26 ± 12.78	42.82 ± 12.56	1.601	0.449
SWS (% of TST)	753	19.50 ± 9.53	15.97 ± 10.00	17.20 ± 8.53	21.792	<0.001
REM (% of TST)	733	20.26 ± 7.51	19.41 ± 8.34	17.21 ± 8.53	14.281	0.001
REM SOL	743	47.03 ± 55.51	59.24 ± 64.23	52.42 ± 65.50	2.316	0.314
SOL	752	9.97 ± 15.20	10.57 ± 30.82	8.61 ± 13.61	2.906	0.234
Mean sleep latency at MSLT	816	3.98 ± 3.06	3.78 ± 3.06	3.79 ± 2.70	1.164	0.559
Number of SOREMP (%)	777					
0–15		4.40	4.40	3.90	0.382	0.826
20–40		21.10	21.60	18.60		
50–75		29.60	32.40	36.10		
80–100		44.90	41.80	41.40		
Hypocretin (%)						
0–40 pg mL^{-1}		80.20	89.80	90.10	5.186	0.75
>40 pg mL^{-1}		19.80	10.20	9.90		

F-value: derived from one-way ANOVA. BMI, body mass index; EDS, excessive daytime sleepiness; ESS, Epworth Sleepiness Scale; HH, hypnagogic or hypnopompic hallucination; K–W, Kruskal–Wallis test for not normally distributed variables; MSLT, multiple sleep latency test; PSG, polysomnography; REM, rapid eye movement; SE, sleep efficiency; SOL, sleep-onset latency; SOREMP, sleep-onset REM period; SP, sleep paralysis; SWS, slow-wave sleep; TST, total sleep time.

Table 4. Significant differences due to sleepiness (EDS: upper, MSLT: lower table)

Variable	n	*ESS 0–10*	*ESS > 10*	t	P
Diagnostic delay	627	7.81 ± 7.04	14.7 ± 14.6	6.488	0.011
SE	691	87.06 ± 11.2	83.47 ± 11.17	5.491	0.019
Mean sleep latency on MSLT	774	5.99 ± 3.94	3.75 ± 2.9	15.307	<0.001
Number of SOREMP (%)	803				
0–15		84.20	0.10	90.849	<0.001
20–40		10.50	21.50		
50–75		NA	34.50		
80–100		5.30	43.80		
Variable	*n*	MSLT <8	MSLT > 8	v^2/t	*P*
Age at EDS onset (years)	870	23.36 ± 12.14	19.8 ± 11.03	6.727	0.009
Age at cataplexy onset (years)	623	26.33 ± 12.93	21.94 ± 11.55	5.305	0.021
ESS	774	17.73 ± 3.69	14.95 ± 4.26	30.978	<0.001
Number of SOREMP (%)	788				
0–15		3.00	9.80	21.293	<0.001
20–40		18.80	40.90		
50–75		33.60	22.90		
80–100		44.70	26.20		

EDS, excessive daytime sleepiness; ESS, Epworth Sleepiness Scale; MSLT, multiple sleep latency test; SE, sleep efficiency; SOREMP, sleep-onset rapid eye movement period

Table 5. Significant differences due to the severity of cataplexy

Variable/frequency of cataplexy	*1*	*2*	*3*	*4*	*5*	*F/K–W*	P
Diagnostic delay	16.67 ± 14.76	18.30 ± 15.84	15.11 ± 14.29	14.88 ± 14.59	11.91 ± 12.82	4.480	0.001
EDS, HH and SP (% positive)	17.50	28.70	42.50	40.60	51.40	10.125	<0.001

F-value: derived from one-way ANOVA. EDS, excessive daytime sleepiness; HH, hypnagogic or hypnopompic hallucination; K–W, Kruskal–Wallis test for not normally distributed variables; SP, sleep paralysis.

Table 6. Clinical variables

Parameter	*n (all)*	*Mean ± SD*	*Men*	*Women*	t-*test*	P
HH (% positive)	587	63.10	58.90	68.00	8.030	0.005
SP (% positive)	488	52.60	50.20	55.50	NS	
HH and SP (% positive)	402	43.60	40.40	47.60	6.622	0.01
EDS, HH and SP (% positive)	301	41.80	38.90	44.60	NS	
ESS score	803	17.45 ± 3.86	17.33 ± 3.91	17.57 ± 3.80	NS	
EDS (% positive)	765	95.30	95.40	95.10	4.488	0.034
Frequency of cataplexy (%)						
1 (one or less cataplexy attacks per year)	48	5.80	6.80	4.60	NS	
2 (more than one cataplexy attacks per year, but less than one per month)	108	13.00	11.40	15.30		
3 (more than one attacks per month, but less than one attack per week)	154	18.60	18.60	18.50		
4 (more than one attack per week, but less than one per day)	172	20.70	19.20	22.60		
5 (at least one cataplexy attack per day)	347	41.90	44.10	39.00		

Table 7. PSG and MSLT variables and hypocretin-1 levels

Parameter	*n (all)*	*All mean ± SD*	*Men*	*Women*	t-*test*	P
TST	751	411.70 ± 80.45	410.33 ± 79.85	414.26 ± 80.38	NS	
SE	848	83.80 ± 11.53	82.86 ± 11.20	84.95 ± 11.82	74 791	<0.001*
Stage 1 (% of TST)	772	14.62 ± 10.36	16.46 ± 10.89	12.96 ± 9.21	55949.9	<0.001*
Stage 2 (% of TST)	773	43.75 ± 12.3	43.04 ± 12.55	44.74 ± 11.91	66203.5	0.03*
SWS (% of TST)	808	17.54 ±9.97	16.36 ± 9.88	18.86 ± 9.89	67725.5	<0.001*
REM (% of TST)	788	19.17± 8.54	19.27 ± 8.16	19.04 ± 8.98	NS	
REM-onset latency	835	54.59 ± 65.30	55.03 ± 67.39	53.92 ± 62.91	NS	
SOL (PSG)	844	10.33 ± 23.80	11.86 ± 28.35	9.30 ± 14.75	NS	
Mean sleep latency (MSLT)	927	3.92 ± 3.03	4.10 ± 3.23	3.67 ± 2.70	NS	
Number of SOREMP (%)						
0–15	34	4.00	3.80	4.30	NS	
20–40	169	20.50	22.90	17.70		
50–75	265	32.10	27.90	36.80		
80–100	358	43.40	45.30	41.30		
Hypocretin (%)						
Undetectable (<40 pg mL^{-1})	254	86.40	55.11	44.89		
(>40 pg mL^{-1})	40	13.60	57.50	42.50		

*Mann–Whitney U-test, t-test for ranks. MSLT, multiple sleep latency test; PSG, polysomnography; REM rapid eye movement; SE, sleep efficiency; SOL, sleep-onset latency; SWS, slow-wave sleep; TST, total sleep time.

Table 8. Principal component analysis

Rotated component matrix	*Component*							
	1	2	3	4	5	6	7	8
BMI	0.312	0.073	0.306	0.039	-0.191	-0.314	-0.210	0.023
Age at diagnostic	0.776	0.109	0.089	0.150	-0.195	0.157	-0.142	-0.074
Diagnostic delay	0.730	0.112	0.102	0.058	-0.198	0.331	-0.134	-0.199
Frequency of cataplexy	-0.027	-0.039	0.069	-0.053	0.733	-0.158	0.008	0.062
TST	-0.749	0.015	-0.076	0.086	-0.148	0.145	-0.153	-0.022
SE	-0.737	0.011	-0.048	0.012	-0.124	0.183	-0.189	-0.028
Stage 1	0.442	-0.045	0.460	-0.203	-0.014	-0.174	-0.248	0.429
Stage 2	-0.178	0.057	0.103	0.869	-0.095	0.124	0.120	-0.226
SWS	-0.279	0.093	-0.056	-0.754	-0.062	0.136	0.137	-0.290
REM	-0.165	0.038	-0.733	-0.178	-0.032	0.040	0.047	-0.028
SOL	0.089	0.008	0.085	0.007	-0.041	-0.047	0.875	0.094
REM latency	-0.002	0.011	0.731	-0.031	0.051	0.184	0.246	-0.173
Mean MSLT	0.061	-0.506	-0.031	0.039	0.193	0.120	-0.043	-0.330
No. SOREMPs	-0.027	0.871	0.026	0.007	0.037	0.014	-0.031	0.057
ESS score	0.098	0.885	-0.049	-0.029	0.049	0.073	-0.054	0.021
EDS	0.107	0.693	-0.025	0.031	0.245	0.041	0.069	-0.094
Hypocretin	-0.138	0.116	-0.125	0.034	0.090	0.228	0.135	0.738
EDS + HH + SP	0.002	0.261	-0.024	0.026	0.730	0.150	-0.044	-0.013
First symptom	0.005	0.038	0.080	0.010	-0.047	0.811	-0.057	0.137

BMI, body mass index; EDS, excessive daytime sleepiness; ESS, Epworth Sleepiness Scale; HH, hypnagogic or hypnopompic hallucination; MSLT, multiple sleep latency test; REM, rapid eye movement; SE, sleep efficiency; SOL, sleep-onset latency; SOREMP, sleep-onset REM period; SP, sleep paralysis; SWS, slow-wave sleep; TST, total sleep time.

Table 9. Genome-wide association analysis of narcolepsy phenotypes

Phenotype	*rs#*	*aA*	*aB*	*x1*	*p1*	*x2*	*p2*	*x_meta*	*p_meta*
ESS (n1 = 418, n2 = 252)	rs16966122	A	G	-2.3655	9.11E-07	-0.8656	6.82E-02	-1.601	2.08E-06
Age EDS onset (n1 = 530, n2 = 319)	rs2859998	A	G	-4.6048	2.33E-07	-3.0064	2.20E-01	-4.4145	1.28E-07
Age EDS onset (n1 = 530, n2 = 319)	rs6072697	C	T	14.8434	4.29E-06	4.4731	2.76E-01	10.8306	1.84E-05
Age cataplexy onset (n1 = 435, n2 = 305)	rs12425451	C	T	-7.9529	7.30E-07	-4.5204	4.83E-02	-6.7866	1.97E-07
HH (n1 = 458, n2 = 310)	rs10160605	A	G	0.8865	3.84E-06	0.0294	8.48E-01	0.363	2.43E-03
TST (n1 = 442, n2 = 180)	rs304468	C	T	-28.7108	1.18E-06	9.1773	2.65E-01	15.7748	1.00E-03
TST (n1 = 442, n2 = 180)	rs890227	A	G	149.2937	4.11E-07	-16.656	3.77E-01	31.3251	4.82E-02
SE (n1 = 451, n2 = 268)	rs1515773	C	T	-5.6438	3.37E-07	3.083	1.80E-01	-3.9944	6.10E-05
SE (n1 = 451, n2 = 268)	rs2426087	A	G	11.638	4.30E-07	-1.9517	7.94E-01	10.463	1.98E-06
SOREMPs (n1 = 490, n2 = 298)	rs9397716	C	T	-11.7672	2.13E-05	0.0022	9.40E-01	0.0009	9.75E-01
SOREMPs (n1 = 490, n2 = 298)	rs9551427	A	G	-10.883	2.28E-05	0.0345	1.32E-01	0.0336	1.41E-01
BMI (n1 = 463, n2 = 307)	rs1882687	A	C	2.1251	3.90E-07	-0.3574	4.95E-01	1.1544	4.13E-04
REMS (n1 = 429, n2 = 260)	rs17294110	A	G	3.0476	8.91E-06	-2.5829	8.55E-02	2.0692	9.07E-04

rs#: SNP; aA and aB: minor and major alleles; x1 and x2: effect size for discovery and replication; meta: metanalysis; n1 and n2: number of patients in discovery and replication analyses. BMI, body mass index; EDS, excessive daytime sleepiness; ESS, Epworth Sleepiness Scale; HH, hypnagogic or hypnopompic hallucination; REMS rapid eye movement sleep; SE, sleep efficiency; SOREMP, sleep-onset REM period; TST, total sleep

Annex II

Running Head: Sleep and cardiovascular risk factors

Objective Sleep Structure and Cardiovascular Risk Factors in the General Population: The Hypnolaus Study

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Conflict of interest

The authors declare no conflict of interest.

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Abstract

Study Objectives: To evaluate the association between objective sleep measures and metabolic syndrome (MS), hypertension, diabetes and obesity.

Design: Cross-sectional study.

Setting: General population sample.

Participants: 2162 subjects (51.2% women, mean age 58.4±11.1).

Interventions: Subjects were evaluated for hypertension, diabetes, overweight/obesity and MS, and underwent a full polysomnography (PSG).

Measurements and Results: PSG measured variables included: Total sleep time (TST), percentage and time spent in slow wave sleep (SWS) and in rapid eye movement (REM) sleep, sleep efficiency and arousal index (ArI). In univariate analyses, MS was associated with decreased TST, SWS, REM sleep, sleep efficiency and increased ArI. After adjustment for age, gender, smoking, alcohol, physical activity, drugs that affect sleep and depression, the ArI remained significantly higher, but the difference disappeared in subjects without significant sleep disordered breathing (SDB). Differences in sleep structure were also found according to the presence or absence of hypertension, diabetes and overweight/obesity in univariate analysis. However, these differences were attenuated after multivariate adjustment and after excluding subjects with significant SDB.

Conclusions: In this population-based sample we found significant associations between sleep structure and MS, hypertension, diabetes and obesity. However, these associations were cancelled after multivariate adjustment. We conclude that normal variations in sleep contribute little if any to MS and associated disorders.

Keywords: Polysomnography, metabolic syndrome, hypertension, diabetes, obesity

Introduction

Cardiovascular diseases (CVD) are the main cause of death worldwide, and are expected to increase in the forthcoming years[1]. Lifestyle factors such as tobacco use, high-fat diet and physical inactivity strongly increase CV risk factors, but they may not fully account for their development[2]. It is therefore important to identify new underlying determinants of CVD.

Recent research identified relationships between sleep quantity and CV risk factors. Numerous population studies, summarized in recent meta-analyses, suggested that sleep duration may be associated with obesity[3], hypertension[4], type 2 diabetes[5], the metabolic syndrome (MS)[6], cardiovascular outcomes (including coronary heart disease, stroke and total CVD)[7] and overall mortality[8]. In particular, for most of these outcomes there seems to be a U-shaped relationship with sleep duration.

Some of the above studies evaluating the association between sleep duration and cardiovascular and metabolic outcomes yielded conflicting results that can be explained by methodological heterogeneity. Caution has been expressed, from different perspectives, about the interpretation of these findings in critical reviews[9, 10]. One important limitation of prior epidemiological studies is that the definition of ‘‘short’’ and ‘‘long’’ sleep varies across studies. More importantly, almost all previous studies are based on subjective assessments of the duration of sleep, generally based on responses to a single question or by questionnaires that have not been validated against objective sleep measures.

Finally, it was suggested that the effect of sleep on CV risk may be mediated by poor sleep quality. For example, difficulties in initiating or maintaining sleep were associated with a greater risk of type 2 diabetes[5]. Again, these conclusions are based on subjective assessments and do not allow the identification of sleep variables directly linked with CV risk outcomes. Laboratory studies in young healthy adults indicated that selective suppression of slow wave

sleep (SWS) without any change in total sleep time affects glucose homeostasis, potentially increasing the risk of type 2 diabetes[11], and can produce a significant reduction in blood pressure (BP) dipping[12]. Even if these findings demonstrate that sleep structure, independently of sleep duration, can play a role in metabolic disorders, they have not been replicated in large general population studies.

Thus, the aim of this study was to explore the association between sleep structure, objectively measured by polysomnography (PSG) and several CV risk factors, specifically MS, hypertension, diabetes and obesity in a large unselected middle-aged general population sample. We analyzed these associations in the entire sample and after excluding subjects with a significant oxygen desaturation index (ODI>15/h), as intermittent hypoxia related to respiratory disturbances (apneas/hypopneas) plays an independent role in the pathophysiology of CVD[13].

Methods

Population sampling

The HypnoLaus Sleep Cohort study is based on the first follow up of the epidemiologic CoLaus/ PsyCoLaus study. Details of the CoLaus/PsyCoLaus study were previously described[14, 15]. Briefly, the CoLaus/Psycholaus study included a random sample of 6733 subjects (range age: 35-75 years) selected from the residents of Lausanne city (Switzerland) between 2003 and 2006. HypnoLaus evaluated the subjective and objective sleep characteristics of the study population. During the first follow up of the cohort, five years after the initial phase, all subjects who responded underwent a new physical (n=5064) and psychiatric (n=4000) examination and were given questionnaires by trained interviewers, which included questions on demographic, medical, and treatment history as well as smoking

and alcohol consumption. Sleep-related complaints and habits were investigated using the Pittsburgh Sleep Quality Index (PSQI)[16], the Epworth Sleepiness Scale (ESS)[17] and the Berlin questionnaire for sleep disordered breathing (SDB)[18].

CoLaus/PsyCoLaus and Hypnolaus were approved by the Ethics Committee of the University of Lausanne and a written informed consent was obtained from all participants at the baseline and the follow-up assessments.

Polysomnography

3051 consecutive subjects were invited to undergo a full night in-home PSG recording. No selection of the subjects was made based on the questionnaires and the investigators were blinded to the questionnaires' results. During a visit at the Center for Investigation and Research in Sleep (Lausanne University Hospital, Switzerland), trained technicians equipped the subjects with the PSG recorder (Titanium, Embla® Flaga, Reykjavik, Iceland) between 5 and 8 PM. All sleep recordings took place in the subjects' home environment and included a total of 18 channels: six electroencephalography, two electrooculography, three surface electromyography (one submental, two for right and left anterior tibialis muscles), one for electrocardiogram, nasal pressure, thoracic and abdominal belts, body position, oxygen saturation and pulse rate.

All PSG recordings were visually scored by two trained sleep technicians (DA and NT) using Somnologica software (Version 5.1.1, by Embla® Flaga, Reykjavik, Iceland) and reviewed by a trained sleep physician (JHR). Random quality checks were performed by a second physician (RH). Quality control for concordance rate between the two PSG scorers was implemented periodically to ensure that both scorers achieved at least a 90% level of agreement for sleep stages and respiratory events and an 85% level of agreement for

arousals[19]. Sleep stages, leg movements and arousals were scored according to the 2007 AASM criteria[20]. The ODI represents the number ≥3% oxygen saturation drops per hour of sleep. Apneas/hypopneas were scored according to the AASM 2013 rules[21]. The average number of apneas/hypopneas per hour of sleep (apnea-hypopnea index [AHI]) was calculated.

Cardiovascular risk factors

BP was measured in triplicate on the left arm and values averaged between the last two readings. Arterial hypertension was defined as a systolic BP (SBP) ≥140 mmHg and/or a diastolic BP (DBP) ≥90 mmHg or current use of antihypertensive medication. A fasting blood sample was collected for various analyses (including glucose, total cholesterol, high-density lipoprotein (HDL) cholesterol and triglycerides). Diabetes was defined as a fasting blood glucose level ≥7 mmol/L (126 mg/dL) or current use of antidiabetic medication. The body-mass index (BMI) was calculated and subjects were classified as overweight if their BMI was between 25 and 30 kg/m^2 and obese if BMI ≥30 kg/m^2. The MS was defined according to the Adult Treatment Panel III report (ATP-III)[22] in the presence of 3 of the following 5 factors: abdominal obesity (waist circumference >102 cm in men and >88 cm in women), elevated triglycerides (≥1.7 mmol/L, >150 mg/dL), reduced HDL cholesterol (<1.03 mmol/L (40 mg/dL) in men and <1.20 mmol/L (50 mg/dL) in women), elevated BP (≥130/≥85 mmHg) (or hypertension), or elevated fasting glucose (≥ 5.6 mmol/L) (or type 2 diabetes mellitus).

Other variables

Smoking habit was self-reported and was dichotomized as current smoker/ex-smoker or never-smoker. Alcohol drinking was dichotomized as currently drinking or no alcohol consumption. Regular exercise was defined as reports of exercising ≥3 times a week. Medication use, recorded at the time of sleep studies, was coded according to the World Health Organization ATC classification (http://www.whocc.no/atcddd). Diagnosis of lifetime

major depressive disorder was assigned according to DSM-IV criteria with information collected using the French translation[23] of the semi-structured Diagnostic Interview for Genetic Studies (DIGS)[24]. PSGs were recorded between Mondays and Fridays. As Saturday is generally considered to be a day off, the day of week (Friday versus other week days) was considered as a covariate.

Statistical analysis

All statistical tests were performed using Stata 11 (StataCorp, College Station, TX, USA). For descriptive statistics, continuous variables were summarized as mean±standard deviation, while categorical variables were summarized as number of subjects and percentages. Descriptive statistics were also used for sleep characteristics of the subjects based on presence or absence of MS, hypertension, diabetes and obesity. Student's t-test or one-way analysis of variance were used to evaluate univariate differences between PSG variables. These included: sleep duration or total sleep time (TST): total minutes of any stage of sleep from sleep onset to morning awakening; percentage of slow wave sleep (SWS): percentage of TST spent in stage N3/SWS; SWS min: time in minutes spent in stage N3/SWS; rapid eye movement (REM) sleep: percentage of TST spent in REM sleep; REM min: time in minutes spent in REM sleep; sleep efficiency: ratio between the total sleep time and time spent in bed; arousal index (ArI): number of arousals divided by hours of TST. These variables are routinely used in clinical practice to describe both sleep continuity and sleep architecture. The PSG variables were considered as continuous variables. SWS min and REM min were log transformed (natural log) for analysis. Although extremes of sleep duration were previously associated with adverse CV risk outcomes, TST was also evaluated as a continuous variable given the low prevalence of long sleepers (0.3% slept more than 10 hours) and short sleepers (8% slept less than 5 hours) in the Hypnolaus study. However we also compared the short sleepers and

long sleepers, stratifying the subjects in 4 groups according to the TST: <6 h, 6-7h, 7-8h, 8+ h. These analyses are presented in supplementary tables.

At a second step, two multivariate models were used: the first was adjusted for age, gender and day of PSG recording; the second model was adjusted for the same variables plus the following additional covariates: smoking, alcohol use, regular exercise, depression and use of medications that affect sleep (hypnotics, benzodiazepines, antidepressants, neuroleptics and/or antihistamines). BMI was included as a covariate for hypertension and diabetes, but not for the MS as BMI is strongly associated with abdominal obesity and overadjustment might occur. A multivariate analysis with subjects stratified in groups according to the TST (<6 h, 6-7h, 7-8h, 8+ h) was also performed, and results are presented as supplementary data.

When studying the association between sleep and cardiovascular morbidity and mortality, SDB constitutes a key phenotype as it is an independent risk factor for hypertension[25] and insulin resistance[26], and is associated with the MS[27]. SDB describes a group of disorders characterized by abnormalities of respiratory patterns during sleep. These abnormalities cause sleep disturbances (secondary to arousals to resume normal ventilation) and/or repetitive oxygen desaturations. Intermittent hypoxia in SDB seems to be a critical factor in the pathophysiology of cardiovascular and metabolic consequences[13]. As the purpose of this study was to determine if sleep structure is associated with CV risk factors independently of the origin of sleep disruption, we analyzed these associations first in the entire group and then, after excluding subjects with a substantial (>15/h) ODI. We choose a cut-off value of 15/h as the The International Classification of Sleep Disorders (ICSD-3) diagnostic criteria for sleep apnea are based on this metric (AHI ≥ 15/hour or ≥ 5/hour with symptoms)[28]. Of note, ODI was also examined as a continuous variable, with the results yielding similar conclusions. For simplicity, we present only the results using the dichotomous ODI variable.

Multiple testing problems are in a wide variety, ranging from testing multiple doses and endpoints jointly, composite endpoint, non-inferiority and superiority, etc.[29]. In this study, we chose to adjust for multivariate adjustment within each MS component, thus allowing a better control of the type I error rate while not overtly reducing the chances of finding clinically important (and statistically significant) associations. We estimated that within each MS component approximately 20 associations would be tested, thus leading to an adjusted p-value of 0.05/20=0.001 (Bonferroni method).

Results

Feasibility and failures

Of the 3051 contacted subjects, 2168 (71.1%) agreed to have a PSG at home. Subjects who agreed to participate were 8.2 years younger and reported less sleepiness than those who declined. BMI (+0.4 kg/m^2) and PSQI scores (+0.3 pts) were slightly higher in those who declined (Cohen's D test effect size 0.09 for both). The Berlin score was similar in both groups. Technical problems resulting in insufficient data for PSG scoring were encountered in 60 cases (2.8%), 54 PSG were repeated and 6 subjects declined to repeat the study, resulting in 2162 participants (51.2% women, mean age 58.4±11.1 years) included in the final analysis (**figure 1**).

General characteristics

Table 1 summarizes the findings for the entire population and in subjects with an ODI ≤15/h *vs.* those with an ODI >15/h. Overall a total of 30.5% individuals had MS, 41.5% hypertension, 9.9% diabetes, 41.1% were overweight (25≤BMI<30 kg/m^2), and 16.5% were obese (BMI ≥30 kg/m^2). The mean TST was 401.2±72.1 min, the time spent in SWS and REM sleep were respectively 19.7±8.4% and 21.8±6.1% of the TST, the sleep efficiency was 84.6±10.9%, the ArI was 21.3±11/hour of sleep, and the AHI was 15.5±16.3/h.

A total of 751 subjects (34.7% of the sample) had a significant ODI (>15/hour of sleep). Subjects with ODI >15/h were older, more frequently male, had higher rates of MS, hypertension, diabetes and overweight/obesity than subjects with an ODI ≤15/h. Concerning PSG characteristics, subjects with ODI >15/h slept less, spent less time in SWS and REM sleep, had lower sleep efficiency, and higher ArI.

Sleep structure and CV risk factors

Tables 2 to 5 show the sleep characteristics of subjects according to the presence or absence of the MS, hypertension, diabetes and overweight/obesity in the overall sample and in subjects without clinically significant desaturation (ODI≤15/h) from models adjusted for age, gender and day of week of the PSG recording (P_1), and smoking, alcohol, physical activity, drugs that affect sleep and depression (P_2).

In bivariate analyses subjects with MS had lower TST, spent less time in SWS and in REM sleep, had poorer sleep efficiency and higher ArI than those without MS. The differences were attenuated after adjusting for age, gender and the day on which the recording was performed (**Table 2**). Finally, only the ArI remained significantly different after additional adjustments (adjusted means: 20.3±0.3 *vs.* 23.3±0.4/h; p<0.001). However the difference lost statistical significance in subjects with an ODI≤15/h (p=0.72).

In the whole group, hypertension was associated with lower TST, decreased SWS and REM sleep, lower sleep efficiency, and higher ArI. Sleep efficiency was different in subjects with hypertension when compared with those without hypertension after adjusting for age, gender and the day of the PSG recording (85.1±0.3 *vs.* 84.2±0.4%; p<0.05), but no significant differences were found between groups concerning sleep structure after adjusting for additional covariates (BMI, smoking, alcohol, physical activity, drugs that affect sleep and depression) (**Table 3**). In subjects without clinically significant ODI, hypertension was

associated with was slightly lower sleep efficiency in the multi-variable adjusted model (adjusted means: 86.9±0.3% *vs.* 85.4±0.5%; p=0.02).

Unadjusted analyses showed significant differences in sleep structure between subjects with diabetes when compared with subjects without: they spent less time in SWS and in REM sleep, had lower sleep efficiency and higher ArI. In the multi-variable adjusted model only TST and sleep efficiency remained slightly different between groups (**Table 4**). In subjects with an ODI≤15/h, after adjusting for potential confounders, the differences showed a marginal trend (p value for TST =0.11; p value for sleep efficiency = 0.07).

Unadjusted comparisons of sleep structure showed that subjects with a BMI>25 kg/m^2 had lower TST, spent less time in SWS and in REM sleep, had poorer sleep efficiency and higher ArI than those with a normal weight. These differences were attenuated in the multi-variable adjusted model, but SWS (20.8±0.3 *vs.* 19.5±0.3%; p=0.002), time spent in SWS (82±1 vs. 78±1 min, p<0.05) and the ArI (19.6±0.4 *vs.* 22±0.3/h; p<0.001) remained different (**Table 5**). In subjects with an ODI≤15/h, the percentage and the time spent in REM sleep were higher in overweight/obese subjects (adjusted means: 22.4±0.2% *vs.* 23.4±0.2%, p=0.002; 91±1 vs 97±1 min, p<0.005), even though these differences were small.

As previous population studies suggest a U-shaped relationship between subjective sleep duration and cardiovascular outcomes, we performed further analysis stratifying the subjects in 4 groups according to the TST: <6 h, 6-7h, 7-8h, 8+ h. These data are presented as supplementary tables (**Supplemental tables 1 and 2**). In this analysis the most consistent finding was that the prevalence of hypertension showed an inverse association with sleep duration for the whole group, and a U-shaped association in subjects without significant ODI (**Supplemental table 1**). However, in the multivariate logistic regression analysis (**Supplemental table 2**) after adjusting for gender, age, smoking, alcohol, physical activity,

drugs that affect sleep, BMI and depression, the odds ratio (95% confidence interval) for hypertension for subjects sleeping less than 6h was 1.19 (0.87 - 1.62) for the whole population and 1.45 (0.97 - 2.16) for those without significant ODI, taking as reference subjects with a sleep duration of 7-8 h. Significant differences between groups were found for the prevalence of the MS in the whole group, as subjects with a TST <6h had a higher prevalence of MS than the other groups (no U-shaped relationship), but this difference was not found in subjects without significant ODI (≤15/h). No significant differences between groups were found in the multivariate logistic regression analysis. No U-shaped association was found between groups concerning the prevalence of diabetes. In the multivariate logistic regression analysis, the OR for diabetes was lower in subjects with a TST less than 6h compared with the reference group (OR 0.57, CI: 0.35 - 0.93) in the whole group, but not in the subjects without significant sleep disordered breathing. Finally, a U-shaped association was found for the prevalence of BMI >25 kg/m^2 in the whole group, but not when considering subjects with ODI ≤15/h. However, in the multivariate logistic regression analysis the OR for a BMI >25 kg/m2 was significantly higher in subjects with a TST more than 8h and no significant ODI, when compared with the reference group (1.63 (1.09 - 2.43)). Overall, these analyses confirm that TST, whether used as a continuous or a categorical variable, does not show consistent associations with CV risk factors.

Discussion

To the best of our knowledge, this one of the largest population-based study assessing the relationship between sleep variables, objectively measured by PSG, and CV risk factors, specifically, the MS, hypertension, diabetes and obesity. In our study the MS was more prevalent in subjects whose sleep was shorter, lighter (as measured by decreased SWS and REM sleep), more fragmented and with lower sleep efficiency. However, these differences

were attenuated when adjusting for potential confounding factors and only the ArI remained significantly higher in subjects with the MS in the multivariate model. This association disappeared in subjects without significant ODI (clinical cutoff of ODI≤15/h), suggesting that the increased ArI in subjects with MS in the whole group could be related to the presence of respiratory disturbances. Another possibility would be a reduction in statistical power due to the decrease in sample size.

Crude unadjusted analyses also showed significant differences in sleep duration and structure between subjects according to the presence or absence of hypertension, diabetes and overweight/obesity. But, as for the MS, these differences disappeared in subjects without significant ODI after controlling for confounders. In a conservative approach to avoid the possibility to find significant differences due to chance we preferred a p-value <0.001 for statistical significance. Choosing a less rigorous p-value of <0.05 leads to a statistically significant association between hypertension and sleep efficiency, in subjects without significant ODI and after adjusting for possible confounding factors, yet, these differences are very small (86.9±0.3% *vs.* 85.4±05%, p=0.02). In the same way, overweight subjects without significant ODI spent more time in REM sleep, but again these differences were small (22.4±0.2% *vs.* 23.4±0.2%, p=0.002; 91±1 *vs.* 97±1, p<0.005). As pointed by others[10], such small differences in epidemiological studies, even if statistically significant, are of questionable clinical value as this association must be the result of sleeping habits for longtime, and can be easily modified by other behavioral factors, as short exercise exposures or dietary changes. Taken together, these findings suggest that normal variation in sleep duration and structure does not seem to be associated with the MS, hypertension, diabetes and obesity.

Indirect evidence suggests that sleep deprivation may trigger biological changes contributing to MS. Laboratory studies demonstrated that reduced sleep amount produces short-term

adverse effects, as changes in circulating levels of leptin and ghrelin, impaired glucose tolerance, increased cortisol secretion, altered growth hormone metabolism, and changes in BP and sympathetic activity[30, 31]. Also, changes in sleep structure, in particular suppression of SWS, without any change in total sleep time, resulted in decreased insulin sensitivity and reduced glucose tolerance[11]. In the same way, Sayk *et al.* showed that selective deprivation of SWS for one night by acoustic stimulation in healthy subjects produces a significant reduction in BP dipping, but no significant changes in morning BP, urine catecholamine excretion, or HR variability[12]. It should be noted that in laboratory studies, the carefully controlled experimental conditions allow changes in sleep patterns (as major shortening of the sleep duration that cannot be tolerated beyond a few days or the complete absence of SWS) that are not experienced in everyday life in the general population. Additionally, in most of these studies subjects are selected on the basis of the absence of sleep disorders and other comorbidities that can have a significant impact on the development of CV risk factors.

Our observations differ from those of previous epidemiological surveys that have identified several associations between sleep and adverse CV risk factors. Almost all epidemiologic studies examining these associations used subjective sleep assessments, such as self-reported sleep duration, which may lead to major methodological issues considering that there is no validated evidence for direct relationships between subjective and objective measures of sleep. The correlation between self-reported and objectively-measured sleep was shown to be, at best, moderate, and biased by systematic over-reporting[32]. Comparing self-reports of sleep duration *vs.* those obtained by wrist actigraphy shows that people subjectively overestimated their sleep by up to an hour, with R^2 values between these two indices being only 0.22. People estimating their sleep at 5 h usually only slept about 4 h, and those estimating 7 h only slept about 6.6 h[32]. Self-perceived sleep duration is likely to be influenced by factors such as sleep

disorders, sociodemographic profile, social demands, or measures time in bed instead of actual sleep duration.

As pointed out by Kurina et al.,[33] in a recent critical review of the studies looking at the association between sleep duration and mortality, another major issue is that different definitions are used for long and short sleep. Short sleep duration in different studies varied from 4 hours or fewer, to fewer than 7 hours per night, and long sleep from more than 8, to 12 or more hours. The reference "normal" category varied from 7 to 9 hours. From some previous studies causal inference is also difficult to draw due to the lack of control for major confounders.

Only a limited number of studies analyzed the relationship between objectively measured sleep patterns by PSG and morbidity-mortality. In a prospective study of 184 older adults, Dew et al[34] showed that short sleepers (<6 h) did not have significantly higher mortality than the rest of the sample. However, increased mortality was associated with sleep latency >30 min, sleep efficiency <80%, and SWS <1%. The percentage of SWS was not associated with mortality after controlling for age, gender, and baseline medical burden and the potential role of other possible confounders, as the presence of sleep-disordered breathing, were not analyzed. Also in older adults a prospective analysis of 784 subjects participating in the Outcomes of Sleep Disorders in Older Men Study revealed that the amount of time spent in SWS was inversely related to the development of incident hypertension independently of sleep duration and sleep-disordered breathing, and after adjusting for age, race and body mass index[35]. From the same cohort, polysomnographic data from 2745 older men also showed a significant inverse association between quartiles of SWS and BMI but the association was attenuated in men with a respiratory disturbance index $\geq$ 15/h[36]. These studies suggest a possible relationship between SWS and hypertension and obesity in selected populations (older men), but they cannot be generalized to the whole population.

Data from the Penn State cohort showed that insomniacs who slept <6 hours had a significantly increased risk of type 2 diabetes[37], hypertension[38] and mortality[39] compared to the "normal sleep duration, no insomnia" group, after adjusting for confounders. Insomnia with objectively measured short sleep duration but not normal variation in sleep seems to constitute a vulnerability sleep-associated phenotype. In a recently published prospective study on this cohort, self-reported short sleep duration, but not objective sleep duration, was associated with a significant increased risk of incident obesity[40]. Again, perception of one's sleep, as a surrogate marker of emotional stress and subjective sleep disturbances rather than objective sleep duration, seems to be the underlying factor beyond the association.

Two previous studies specifically analyzed the association between sleep and MS using PSG. Nock et al[41] analyzed PSG data from 533 adults participating in the Cleveland Family Sleep Study. The subjects were selected from families with siblings having extreme high or low values for AHI. They found that sleep disturbance was a significant component of MS. They defined a sleep disturbance factor that included 4 measures: the AHI, the ArI, the percentage of sleep time when oxygen saturation was less than 90%, and the SWS%. Again, disturbances in sleep but not normal sleep were identified as the risk factors. More recently, Hall et al[42] analyzed the association between sleep and MS in a group of 368 multi-ethnic middle aged (mean age 51 years) women participating in the SWAN Sleep Study. They analyzed various sleep parameters derived from PSG including EEG power spectral analysis. In bivariate analyses, MS was associated with decreased sleep duration and efficiency and increased NREM beta power and AHI. When entered simultaneously in a multi-variable adjusted model to evaluate their independent contributions, only sleep efficiency and AHI appeared to be independent correlates of MS. Respiratory disturbances seem to be clearly associated with MS, as evidenced in our and in previous studies[27, 43], but the sleep efficiency is unrelated to

MS in our study after adjusting for confounders. Nevertheless, we did find a non statistically significant association between hypertension and sleep efficiency, which was lower in hypertensive subjects. One main explanation for this discrepancy is that we included men and women in our study, while Hall et al.[42] only included women. In addition, our subjects were older and had a higher AHI.

Strengths and limitations

The major strengths of our study is its population-based design, the large sample size, the availability of detailed information on a number of potential confounders and the use of PSG to obtain objective measures of sleep structure and sleep co-morbidities as SDB. Nevertheless, we acknowledge potential limitations. Firstly, our results are based on a single PSG, and even if the PSG is considered the gold standard for sleep studies, a single recorded night may not fully capture the complexity of a phenomenon like sleep. Night-to-night variability in sleep is observed in subjects who undergo PSG related to the so called "first night effect", which can be due to discomfort caused by electrodes, limitation of movements, and the unfamiliar environment of the sleep laboratory[44]. Thus, in order to limit the impact of the first night effect, we performed the PSG at home under "habitual" sleeping environment, and subjects were instructed to maintain their usual sleeping habits. Further, we repeated the PSG in a randomly selected sample of 20 Hypnolaus participants to determine the short-term variability of 2 nights of home-PSG. Only the percentage of TST spent in REM sleep was marginally different between nights (21.4±6.7 *vs.* 24±5%, p=0.04, **Supplemental Table 3**). Repeated home-PSG in the Sleep Heart Health Study cohort revealed similar results, with no evidence of a major "first-night effect"[45]. Secondly, the Hypnolaus study is a monocentric study allowing uniform collection, processing, and analysis of the data, but it is limited to middle aged and elderly Lausanne residents; thus, the results may not be easily extrapolated

to other populations. Finally, this is a cross sectional study and future prospective studies are needed to build upon the present findings.

In conclusion, in this population-based study, we found significant associations between sleep duration and quality and the MS, hypertension, diabetes and obesity. However, these associations are not independent of other known CV risk factors, as age, gender, sedentary life style, obesity, the ODI, depression, smoking, alcohol, or use of medications that affect sleep. We conclude that normal variations in sleep in adults seem to contribute little if any to MS, hypertension, diabetes and obesity.

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Figure 1: Studied population

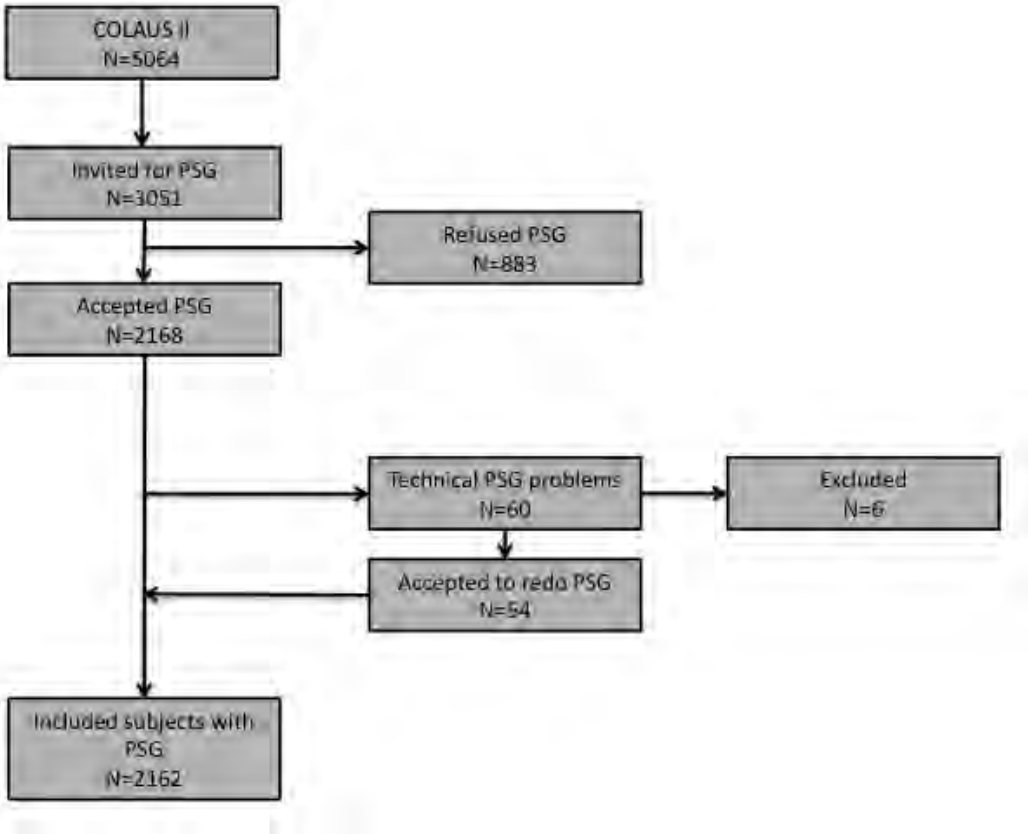

Table 1. Demographic, clinical and polysomnographic characteristics of the participants

	ALL	Subjects with ODI ≤15/h	Subjects with ODI>15/h	P value §
N	2162 (100%)	1411 (65.3%)	751 (34.7%)	
Age, years	58.4 ± 11.1	56.3 ± 10.6	62.8 ± 10.8	<0.001
Gender female	1106 (51.2%)	866 (61.4%)	240 (31.9%)	<0.001
Metabolic syndrome	659 (30.5%)	284 (20.1%)	375 (50.0%)	<0.001
Hypertension	897 (41.5%)	462 (32.7%)	435 (58.0%)	<0.001
Diabetes	214 (9.9%)	82 (5.8%)	132 (17.5%)	<0.001
BMI, kg/m^2	26.2 ± 4.4	25.0 ± 3.9	28.5 ± 4.4	<0.001
<25	909 (42.3%)	764 (54.3%)	145 (19.3%)	<0.001
25-30	884 (41.1%)	509 (36.2%)	375 (49.9%)	<0.001
>30	356 (16.5%)	134 (9.5%)	222 (29.5%)	<0.001
Smokers/ex-smokers	1233 (58.2%)	785 (56.7%)	448 (61.2%)	< 0.05
Regular alcool consumption	1686 (78.0%)	1078 (76.4%)	608 (80.9%)	< 0.01
Sedentary	1047 (55.7%)	662 (52.7%)	385 (61.7%)	<0.001
Taking drugs that influence sleep*	400 (18.5%)	250 (17.7%)	150 (20.0%)	< 0.01
Depression (lifetime or current)	284 (14.8%)	184 (14.6%)	100 (15.1%)	< 0.05
TST, min	401.2 ± 72.1	406.5 ± 45.3	391 ± 74.2	<0.001
SWS %	19.7 ± 8.4	21.0 ± 8.1	17.2 ± 8.4	<0.001
SWS, min	78.6 ± 34.8	84.6 ± 33.1	67.1 ± 35.1	<0.001
REM %	21.8 ± 6.1	22.7 ± 5.7	20.2 ± 6.6	<0.001
REM, min	88.6 ± 31.1	93.2 ± 29.8	80.1 ± 31.6	<0.001
Sleep efficiency %	84.6 ± 10.9	86.1 ± 9.9	81.7 ± 11.9	<0.001
Arousal index, n/h	21.3 ± 11.0	17.6 ± 7.9	28.3 ± 12.6	<0.001
AHI, n/h	15.5 ± 16.3	6.9 ± 14.9	31.8 ± 17.6	<0.001
ODI n/h	14.7 ± 15.2	6.4 ± 10.6	30.5 ± 16.1	<0.001

Results are expressed as N (%) or mean ± standard deviation. BMI: body mass index; TST: Total sleep time/ Sleep duration; SWS%: Percentage of TST spent in slow wave sleep/ stage N3; SWS min: time in minutes spent in stage N3/SWS; REM sleep: percentage of TST spent in REM sleep; REM min: time in minutes spent in REM sleep; Sleep efficiency: TST / time spent in bed × 100; ArI: arousal index; AHI: apnea/hypopnea index; ODI: oxygen desaturation (>3%) index.* Drugs that

influence sleep were considered as “present” if participants were using benzodiazepines or derivates (ATC codes: N05BA, N05CD, N03AE), hypnotics (N05CF), antidepressants (N06A), neuroleptics (N05A) or antihistaminics (R06A).

§ Comparing ODI≤15/h vs. ODI>15/h.

Table 2: Sleep characteristics according to the absence or presence of the metabolic syndrome, a) in the whole population, b) in subjects without significant ODI (≤15/h)

a)

	Absent	Present		Absent	Present	
Group size	**1503**	**659**	**P_1**	**1503**	**659**	**P_2**
	(69.5%)	**(30.5%)**		**(69.5%)**	**(30.5%)**	
Total sleep time (min)	401 ± 2	401 ± 3	0.79	400 ± 2	401 ± 3	0.72
SWS (%)	20.0 ± 0.2	19.3 ± 0.3	0.07	20.3 ± 0.2	19.7 ± 0.4	0.18
SWS (min) §	80 ± 1	77 ± 1	0.12	81 ± 1	78 ± 1	0.42
REM (%)	22.2 ± 0.2	21.4 ± 0.2	<0.01	22.2 ± 0.2	21.7 ± 0.3	0.09
REM (min) §	90 ± 1	87 ± 1	0.04	90 ± 1	88 ± 1	0.32
Sleep efficiency (%)	84.9 ± 0.3	84.3 ± 0.4	0.19	84.8 ± 0.3	85.1 ± 0.4	0.57
Arousal index (n/h) §	20.3 ± 0.3	23.3 ± 0.4	<0.001	20.2 ± 0.3	22.9 ± 0.5	<0.001

b)

	Absent	Present		Absent	Present	
Group size	**1127**	**284**	**P_1**	**1127**	**284**	**P_2**
	(79.9%)	**(20.1%)**		**(79.9%)**	**(20.1%)**	
Total sleep time (min)	406 ± 2	409 ± 4	0.49	405 ± 2	409 ± 5	0.39
SWS (%)	21.1 ± 0.2	21.1 ± 0.5	0.99	21.3 ± 0.3	21.4 ± 0.6	0.89
SWS (min) §	85 ± 1	85 ± 2	0.99	85 ± 1	86 ± 2	0.78
REM (%)	22.8 ± 0.2	22.5 ± 0.3	0.45	22.8 ± 0.2	22.9 ± 0.4	0.97
REM (min) §	93 ± 1	94 ± 2	0.90	93 ± 1	95 ± 2	0.48
Sleep efficiency (%)	86.2 ± 0.3	86.4 ± 0.6	0.73	86.2 ± 0.3	87.3 ± 0.6	0.12
Arousal index (n/h) §	17.4 ± 0.2	17.5 ± 0.5	0.97	17.4 ± 0.2	17.1 ± 0.5	0.72

Results are expressed as multivariable adjusted mean ± standard error. P_1 : adjusted for gender, age and day of week (Friday) ; P_2 : adjusted for gender, age, day of week (Friday), smoking, alcohol, physical activity, drugs that affect sleep and depression. § between-group comparisons performed using log-transformed data. Statistical analysis by analysis of variance. α level set at P < 0.001.

Table 3: Sleep characteristics according to the absence or presence of hypertension, a) in the whole population, b) in subjects without significant ODI (≤15/h)

a)

	Absent	Present		Absent	Present	
Group size	**1263**	**897**	**P_1**	**1263**	**897**	**P_2**
Total sleep time (min)	402 ± 2	400 ± 3	0.53	402 ± 2	398 ± 3	0.31
SWS (%)	19.9 ± 0.2	19.7 ± 0.3	0.57	20.1 ± 0.3	20.1 ± 0.3	0.88
SWS (min) §	80 ± 1	78 ± 1	0.24	80 ± 1	79 ± 1	0.44
REM (%)	22.1 ± 0.2	21.8 ± 0.2	0.33	22.1 ± 0.2	21.9 ± 0.2	0.53
REM (min) §	90 ± 1	88 ± 1	0.19	90 ± 1	88 ± 1	0.23
Sleep efficiency (%)	85.1 ± 0.3	84.2 ± 0.4	<0.05	85.2 ± 0.3	84.5 ± 0.4	0.16
Arousal index (n/h) §	20.8 ± 0.3	21.7 ± 0.4	0.24	20.7 ± 0.3	21.2 ± 0.4	0.77

b)

	Absent	Present		Absent	Present	
Group size	**949**	**462**	**P_1**	**949**	**462**	**P_2**
Total sleep time (min)	409 ± 2	401 ± 3	<0.05	408 ± 2	400 ± 4	0.08
SWS (%)	21.1 ± 0.3	21.3 ± 0.4	0.74	21.2 ± 0.3	21.6 ± 0.4	0.49
SWS (min) §	86 ± 1	84 ± 2	0.28	86 ± 1	85 ± 2	0.64
REM (%)	22.8 ± 0.2	22.7 ± 0.3	0.90	22.9 ± 0.2	22.8 ± 0.3	0.79
REM (min) §	94 ± 1	92 ± 1	0.23	94 ± 1	92 ± 2	0.26
Sleep efficiency (%)	86.8 ± 0.3	85.1 ± 0.5	<0.005	86.9 ± 0.3	85.4 ± 0.5	0.02
Arousal index (n/h) §	17.7 ± 0.3	16.9 ± 0.4	0.17	17.7 ± 0.3	16.6 ± 0.4	0.09

Results are expressed as multivariable adjusted mean ± standard error. P_1 : adjusted for gender, age and day of week (Friday) ; P_2 : adjusted for gender, age, day of week (Friday), smoking, alcohol, physical activity, body mass index, drugs that affect sleep and depression. § between-group comparisons performed using log-transformed data. Statistical analysis by analysis of variance. α level set at $P < 0.001$.

Table 4: Sleep characteristics according to the absence or presence of diabetes, a) in the whole population, b) in subjects without significant ODI (≤15/h)

a)

	Absent	Present		Absent	Present	
Group size	**1948**	**214**	**P_1**	**1948**	**214**	**P_2**
Total sleep time (min)	401 ± 2	407 ± 5	0.20	399 ± 2	413 ± 6	0.02
SWS (%)	19.9 ± 0.2	19.3 ± 0.6	0.32	20.1 ± 0.2	20.2 ± 0.7	0.92
SWS (min) §	79 ± 1	77 ± 2	0.25	80 ± 1	81 ± 3	0.58
REM (%)	22.0 ± 0.1	21.2 ± 0.4	0.06	22.1 ± 0.1	21.7 ± 0.5	0.43
REM (min) §	89 ± 1	87 ± 2	0.86	89 ± 1	90 ± 2	0.25
Sleep efficiency (%)	84.7 ± 0.2	85.0 ± 0.7	0.65	84.8 ± 0.2	86.5 ± 0.8	0.04
Arousal index (n/h) §	20.9 ± 0.2	24.0 ± 0.7	<0.005	20.9 ± 0.2	21.8 ± 0.8	0.30

b)

	Absent	Present		Absent	Present	
Group size	**1329**	**82**	**P_1**	**1329**	**82**	**P_2**
Total sleep time (min)	406 ± 2	413 ± 8	0.38	405 ± 2	419 ± 8	0.11
SWS (%)	21.1 ± 0.2	21.6 ± 0.9	0.64	21.3 ± 0.2	20.9 ± 1.0	0.69
SWS (min) §	85 ± 1	86 ± 4	0.81	85 ± 1	85 ± 4	0.61
REM (%)	22.8 ± 0.2	21.7 ± 0.6	0.08	22.9 ± 0.2	21.8 ± 0.7	0.13
REM (min) §	94 ± 1	92 ± 3	0.95	94 ± 1	93 ± 4	0.70
Sleep efficiency (%)	86.2 ± 0.3	86.9 ± 1.1	0.58	86.3 ± 0.3	88.4 ± 1.1	0.07
Arousal index (n/h) §	17.4 ± 0.2	17.5 ± 0.9	0.83	17.3 ± 0.2	17.7 ± 1.0	0.67

Results are expressed as multivariable adjusted mean ± standard error. P_1 : adjusted for gender, age and day of week (Friday) ; P_2 : adjusted for gender, age, day of week (Friday), smoking, alcohol, physical activity, body mass index, drugs that affect sleep and depression. § between-group comparisons performed using log-transformed data. Statistical analysis by Student's t-test or analysis of variance. α level set at $P < 0.001$.

Table 5: Sleep characteristics according to the absence or presence of overweight/obesity (body mass index ≥ 25 kg/m^2), a) in the whole population, b) in subjects without significant ODI (≤15/h)

a)

	Absent	Present		Absent	Present	
Group size	**909**	**1240**	**P_1**	**909**	**1240**	**P_2**
Total sleep time (min)	399 ± 2	402 ± 2	0.27	399 ± 2	401 ± 2	0.46
SWS (%)	20.7 ± 0.3	19.2 ± 0.2	<0.001	20.8 ± 0.3	19.5 ± 0.3	0.002
SWS (min) §	82 ± 1	77 ± 1	<0.01	82 ± 1	78 ± 1	<0.05
REM (%)	21.9 ± 0.2	22.0 ± 0.2	0.62	21.8 ± 0.2	22.3 ± 0.2	0.09
REM (min) §	88 ± 1	90 ± 1	0.34	88 ± 1	90 ± 1	0.09
Sleep efficiency (%)	85.0 ± 0.3	84.5 ± 0.3	0.28	85.0 ± 0.4	84.9 ± 0.3	0.80
Arousal index (n/h) §	19.5 ± 0.3	22.4 ± 0.3	<0.001	19.6 ± 0.4	22.0 ± 0.3	<0.001

b)

	Absent	Present		Absent	Present	
Group size	**764**	**643**	**P_1**	**764**	**643**	**P_2**
Total sleep time (min)	403 ± 2	410 ± 3	0.06	403 ± 3	409 ± 3	0.12
SWS (%)	21.6 ± 0.3	20.6 ± 0.3	<0.05	21.7 ± 0.3	20.8 ± 0.4	0.06
SWS (min) §	86 ± 1	84 ± 1	0.23	86 ± 1	84 ± 1	0.37
REM (%)	22.5 ± 0.2	23.1 ± 0.2	<0.05	22.4 ± 0.2	23.4 ± 0.2	0.002
REM (min) §	92 ± 1	96 ± 1	<0.01	91 ± 1	97 ± 1	<0.005
Sleep efficiency (%)	86.3 ± 0.3	86.2 ± 0.4	0.90	86.3 ± 0.4	86.5 ± 0.4	0.63
Arousal index (n/h) §	17.0 ± 0.3	17.9 ± 0.3	0.09	17.1 ± 0.3	17.6 ± 0.3	0.47

Results are expressed as multivariable adjusted mean ± standard error. P_1 : adjusted for gender, age and day of week (Friday) ; P_2 : adjusted for gender, age, day of week (Friday), smoking, alcohol, physical activity, drugs that affect sleep and depression. § between-group comparisons performed using log-transformed data. Statistical analysis by Student's t-test or analysis of variance. α level set at $P < 0.001$.

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